Glucose Transport In Developing Ehrlich Ascites Tumor Cells

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B. Sc. (Hon.), C.U.H.K.

A THESIS SUBMITTED IN PARTIAL FULFILMENT

OF THE REQUIREMENT FOR THE DEGREE

OF MASTER OF PHILOSOPHY

IN

BIOCHEMISTRY

June, 1983

THE CHINESE UNIVERSITY OF HONG KONG

This thesis is especially dedicated to my mother.

We accept this thesis as comfirming to the required

standard for the degree of

MASTER OF PHILOSOPHY

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ABSTRACT

The ability of Ehrlich ascites tumor cells to take up glucose increased progressively during the course of tumor development *in vivo*. Simultaneously as the rate of glucose uptake rose, the density of glucose-reversible binding sites for cytochalasin B on cell membrane was also found increased.

In its stereospecificity requirement towards competing sugars and in its sensitivity to phloretin and diethylstilbestrol, this class of glucose-sensitive cytochalasin B binding sites resembled the putative glucose carriers identified in various other cell systems and may represent the glucose transporters of Ehrlich ascites tumor cells.

Glucose carriers on tumor cells showed less content in streptozotocin-induced diabetic mice and higher content in insulin-induced hypoglycaemic mice. For *in vitro* studies, insulin had no effect on the content of glucose carriers while glucagon and glucocorticoid could reduce the carrier content in Ehrlich ascites tumor cells. In another *in vitro* studies, low glucose concentration in the medium could increase the glucose carrier number, and by contrast, high glucose concentration in medium could decrease the number of glucose carriers in Ehrlich ascites tumor cells.

Methotrexate arrested the tumor growth, inhibited glucose uptake and reduced the number of glucose carriers in Ehrlich ascites tumor cells *in vivo*. In both methotrexate-treated and untreated cells, the magnitude of changes in number of glucose carriers closely paralleled and sufficiently accounted for the magnitude of changes in glucose

uptake. Qualitative changes in the turnover and affinity for substrate of the carrier molecules and of transport process need not be invoked.

ACKNOWLEDGEMENTS

I would like to express my appreciation to the Department of Biochemistry, The Chinese University of Hong Kong for the provision of facilities required during the investigation. I would also like to express my sincere gratitude to Dr. K.P. Fung for his valuable guidance and discussion throughout the entire period of this investigation. I am also grateful to Dr. C.Y. Lee for his deliberative discussion. Thanks are also due to Professor T.B. Lo and Dr. Y.M. Choy for their willingness to serve as members of my thesis committee. The Financial support from the World Health Foundation (Hong Kong) is acknowledged.

ABBREVIATION

Bo	Total binding sites
CB	Cytochalasin B
EAT	Ehrlich ascites tumor
K_d	Apparent dissociation constant
K_M	Apparent half-saturation constant
MTX	Methotrexate
Vo	Initial uptake rate
Vmax	Maximal uptake rate

CONTENT

INTRODUCTION

I. Glucose metabolism in tumor cells

Tumor cells usually have a characteristically different glucose metabolism from those of normal cells, in that they metabolize glucose mainly via glycolysis even in the presence of oxygen (Warburg 1956). This phenomenon is termed aerobic glycolysis. Whether high aerobic glycolysis is essential for tumor growth or related to the malignant transformation has not yet been clearly established.

However Eltzina (Eltzina 1960) showed that respiration alone was able to maintain in cancer cells an optimal rate of ATP resynthesis and to ensure energetically amino acid incorporation into their protein to the same extent as glycolysis (Eltzina 1953). Shapot (Shapot 1972) suggested that in the tumor bearing organism, the actual condition for optimal respiration can be far from favorable. For example, the pO_2 in various human tumor of skin of more than 200 patients was found to be 25% or less of that of the simultaneously examined normal adjecent skin (Urbach 1956). Oxygen even was apparently absent in the ascites fluid of Ehrlich ascites tumor *in vivo* (Warburg and Hiepler 1952) and almost anaerobic condition was maintained in the ascites fluid *in vivo* (Shapot 1965). When oxygen was introduced intraperitoneally into the mice bearing Ehrlich ascites tumor, the concentration of lactic acid in the ascites fluid dropped gradually and glucose appeared (Shapot 1972). This implied, *in vivo*, respiration of cancer cells under usual conditions is so weak because oxygen supply was insufficient.

Only an extra oxygen supply enabled the cancer cells to undergo respiration and thereby partially blocked glycolysis thus reduced the glucose consumption. However, many evidences (Warburg 1956; Racker 1976) showed that tumor cells metabolized glucose to lactic acid even in the presence of oxygen. The precise mechanism of aerobic glycolysis in the tumor cells remains unknown.

Another characteristically different glucose metabolism in tumor cell is that tumor cells have higher glucose uptake rate than normal cell types (Stein 1967; Fain 1964; Corfford and Renold 1965; Crane *et al* 1957; Saha and Coe 1967; Kolber and LeFevre 1967). For example, Ehrlich ascites tumor cells from *in vitro* culture (Kaminskas 1979) or washed from the peritoneal cavities of mice had higher glucose uptake rate than normal cells (Crane, Field and Cori 1957; Saha and Coe 1967; Rubin 1971). Chick embryo fibroblasts infected with Rous sarcoma virus was also found to be characterized by a higher uptake rate of 2-deoxy-D-glucose accompanied with the transformation than normal uninfected cells growing at the same rate (Martin *et al* 1971).

It was found that glucose uptake in various tumor and normal cell lines including Ehrlich ascites tumor cells is mediated through two distinglished mechanism, namely unsaturable diffusion and saturably facilitated diffusion (Saha and Coe 1967; Kolber and LeFevre 1967; Lieb and Stein 1970; Renner, Plagemann and Bernlohr 1972; Plagemann and Richey 1974; Hantanaka 1974). In general, unsaturable diffusion is caused

by simple diffusion of glucose from extracellular environment into intracellular environment due to the gradient between these two environments. The saturably facilitated diffusion is a carrier-mediated uptake process and follows Michaelis-Menten kinetics.

II. The use of Ehrlich ascites tumor cells as the experimental tumor model

In the following experiments, the tumor model employed is Ehrlich ascites tumor in mice. The origin of Ehrlich ascites tumor is a solid line of Ehrlich carcinoma, an epithetial mammary carcinoma of mice. It was first converted into ascites form by Lowenthal in 1932 and afterwards this tumor model was widely used in cancer research (Yoshida 1971). The subline of Ehrlich ascites tumor we used is Ny Klein which was converted from solid Ehrlich carcinoma by Klein and Klein (1956) into the ascites form and used as the tetraploid Ehrlich ascites tumor. Since this tumor cell line is easy to handle and maintain either *in vivo* or *in vitro* and has the advantage of its ascites nature, it becomes one of the most common tumor cell lines in cancer research.

Ehrlich ascites tumor cells metabolized glucose to lactic acid which was then transported out of the tumor cells (Warburg 1956). The glycolytic capacity of the tumor cells may be responsible for the low glucose concentration (< 0.2 mM) found in the ascites fluid *in vitro* (Fishman and Baily 1974), although it may also be attributable in part to a compromised rate of

glucose delivery by the systemic circulation relative to the dense cell suspension in the peritoneal cavities (Warburg 1956). It was found that the aerobic glycolysis in Ehrlich ascites tumor cells is accompanied with the derangements of phosphofructokinase (Lazo and Sols 1979); pyruvate dehydrogenase complex (Lazo and Sols 1980a); hexokinase (Lazo *et al* 1978; Gregory and Bose 1979); lactate dehydrogenase (Lazo and Sols 1980b); (Na^+, K^+) - ATPase (Scholnick, Lang and Racker 1973) and pyruvate kinase (Gosalvez *et al* 1975). Lazo and Sols (1980b) also found that Ehrlich ascites tumor cells to have a previously unrecognized isoenzyme of pyruvate dehydrogenase. These observation were suggested to be responsible for the aerobic glycolysis in Ehrlich ascites tumor cells, however none of them can accurately explain its actual role in the mechanism of aerobic glycolysis in Ehrlich ascites tumor cells.

III. Investigation of glucose carrier by cytochalasin B binding

Cytochalasin B is a mold metabolite and has been shown to prevent motility and cytokinesis in fibroblastic cells (Carter 1967) by destroying the integrity of the microfilaments in the contractile ring (Schroeder 1969). Weasel *et al* (1971) has further found that cytochalasin B can break down the microfilaments in several cell types and prevent morphogenesis. In addition to its effects on cellular morphology and microfilament structure, cytochalasin B was recently found to be a potent, reversible inhibitor of sugar uptake in cultured cells (Kletzien, Perdue and Springer 1972; Czech, Lynn and Lynn 1973).

It does not prevent the uptake of thymidine, uridine, α-amino-isobutyric acid or calcium in cells (Klatzien, Perdue and Springer 1972; Czech, Lynn and Lynn 1973). Two classes of binding sites for cytochalasin B with different affinities were found in bovine red blood cells, Hela cells and SV40 transformed mouse fibroblasts (Lin, Santi and Spudich 1974). Recently, Jung and Rampal (1977) found that the site I of the three major cytochalasin B binding sites on human erythrocyte and its membrane preparation is dircetly associated with the glucose transport carrier. They later found that the site III was also glucose-sensitive when site II either bound a ligand or was removed (Pinkofsky *et al* 1978), and the binding activities of erythrocytes membrane were partially solubilized.

IV. Glucose carrier on erythrocytes and other cell lines

The nature of glucose-sensitive cytochalasin B binding sites on human erythrocytes were extensively studied. It was found that this cytochalasin B binding site is responsible for glucose transport in erythrocytes (Jung and Rampal 1977; Pinkofsky *et al* 1978;). Deves and Krupka (1978) demonstrated that cytochalasin B can inhibit glucose transport in human erythrocytes by competing with glucose for the carrier on the inner surface of the cell membrane, but there is no cytochalasin B binding site associated with the outward-facing form of the carrier. Meanwhile, cytochalasin B binding component of human erythrocyte monosaccharides transport system has been purified (Kasahara and Hinkle 1977; Bladwin, Bladwin, Gorga

and Lienhard 1979). Kasahera and Hinkle (1977) found that all sugars which inhibit glucose transport can also inhibit the binding of cytochalasin B to this purified transporter. This glucose carrier is a glycoprotein with the apparent molecular weight estimated by SDS PAGE of about 55,000. After the removal of its carbohydrate moiety, its molecular weight is about 46,000 (Kasahara and Hinkle 1977; Sogin and Hinkle 1978;Jung *et al* 1979; Cuppoletti and Jung 1981). This transporter molecule was found heterogeneously glycosylated as they showed a broad zone in SDS PAGE (Gorga, Bladwin and Lienhard 1979). Sogin and Hinkle (1978) showed that their preparation of red cell glucose carrier contains 24% acidic amino acid, and 5% neutral sugars (of which 3% was galactose), 7% glucosamine and 5% sialic acid. This glucose carrier was found to be stereo-specific that it cannot bind L-glucose (Zoccoli, Bladwin and Lienhard 1978). Recently reconstitution of purified human erythrocytes glucose carrier in an artificial membrane (liposome) was used in the studies of the nature of this transporter molecule and found that the reconstituted glucose carrier retained its glucose transport activity and cyto-chalasin B binding activity (Kasahara and Hinkle 1977; Bladwin, Gorga and Lienhard 1981; Lundahl *et al* 1981). Most recently, photoaffinity labelling of glucose carrier with ^{3}H-cytochalasin B was successful in human erythrocyte membrane (Carter-Su *et al* 1982; Shanahan 1982). This finding provides a new technique tool for the study of glucose carrier in other cell lines.

Studies on the glucose carrier of other cell lines are very

limited. Plagemann and his coworkers (Plagemann *et al* 1981; Graff, Wohlhueter and Plagemann 1981) showed that mammalian cell lines possess hexose transport system with directional symmetry but differential mobility of loaded and empty carriers. Several workers used the technique of cytochalasin B to study the effect of insulin on adipocytes. They found that insulin can increase cytochalasin B binding sites as well as glucose transport activitiy in adipocytes and this increase in glucose carriers is due to the translocation of the transporter molecules from microsomal fraction to plasme membrane (Suzuki and Kono 1980; Karnieli *et al* 1981; Kono *et al* 1981; Rash 1982).

Recently, human erythrocytes glucose carrier antibodies were used to determine the distribution of glucose carriers on the membrane of other cell lines (Sogin and Hinkle 1980 a & b; Bladwin and Liebhard 1980).

These antibodies can cross-react with the glucose carreirs of adipocytes (Lienhard *et al* 1982; Wheeler *et al* 1982) and those of normal and Rous sarcoma virus-transformed chick embryo fibroblasts (Salter *et al* 1982). Based on the above evidences, it was suggested that there may be structural similarities shared by the glucose carriers in different cell lines.

V. Glucose-sensitive cytochalasin B binding sites on Ehrlich ascites tumor cells

In 1967, Kolber and Lefever (1967) were the first who demonstrated the evidence for carrier-mediated glucose uptake

by Ehrlich ascites tumor cells. However, in spite of the large accumulation of information on the studies of the nature of glucose carrier on human erythrocytes and other cell lines, the study on glucose carrier on Ehrlich ascites tumor cells is scarce. Cuppoletti *et al* (1981) were the first who showed that cytochalasin B can competitively inhibit the carrier-mediated glucose uptake by Ehrlich ascites tumor cells with an inhibition constant (Ki) of approximately 5×10^{-7} M. They found that cytochalasin B can bind on Ehrlich ascites tumor cells with three different degree of affinities, low affinity with $K_d = 1 \times 10^{-6}$ M, medium affinity with $K_d = 2\text{-}6 \times 10^{-7}$ M and high affinity with $K_d = 2\text{-}6 \times 10^{-8}$ M. The binding of cytochalasin B to the medium affinity binding sites can be completedly inhibited by the addition of glucose and this class of cytochalasin B binding sites was suggested to be responsible for the carrier-mediated glucose uptake which is inhibited by cytochalasin B, and this binding sites is stereospecific for D-glucose. This glucose-sensitive cytochalasin B binding site represents approximately 60% of the total saturable cytochalasin B binding sites on Ehrlich ascites tumor cells. Ehrlich ascites tumor cell is unique among other cell lines in its high content of this binding site which amounts to more than 20% of the plasma membrane protein. It is at least 4-fold greater than that of transformed chicken embryo fibroblasts (Salter and Weber 1979), that of human erythrocytes (Jung and Rampal 1977) and those of many other animal cell lines reported in

literatures (Plagemann, Graff and Wohlhueter, 1977). It is of value to study the role of this high content of glucose-sensitive cytochalasin B binding sites on Ehrlich ascites tumor cells.

Based on the finding of Cuppoletti, Mayhew and Jung (1981), it is reasonable to believe that the glucose-sensitive cytochalasin B binding sites on the Ehrlich ascites tumor cells as well as those of other cell lines (Lin, Santi and Spudich 1974; Jung and Rampal 1977; Pinkofsky *et al* 1978) are closely related or even identical to the glucose carriers which are responsible for the carrier-mediated uptake of glucose.

Since Ehrich ascites tumor cell is characteristic for its high content of glucose-sensitive cytochalasin B binding sites, it provides as a convenient source for the isolation, purification and characterization of this class of glucose-sensitive cytochalasin B binding sites. Preliminary studies has been reported by Cuppoletti, Mayhew and Jung (1981) that this glucose-sensitive cytochalasin B binding site may be a protein.

Study on glucose-sensitive cytochalasin B binding sites on Ehrlich ascites tumor cells is very limited. Therefore it is worthwhile to furhter study its properties especially its binding specificity . It is of significance to determine whether the number of glucose-sensitive cytichalasin B binding sites on Ehrlich ascites tumor cells and other cell lines share the common structures.

VI. Anti-tumor effect of methotrexate on Ehrlich ascites tumor

Methotrexate (MTX) is an analogue of the vitamin folic acid. Folic acid itself is not biologically active and must be reduced by folate reductase and then dihydrofolate reductase to dihydrofolic acid and thence to tetrahydrofolic acid, which accepts 1-carbon fragment from various sources to generate the folate coenzymes. The latter are utilized as 1-carbon group donors in a variety of reactions, including the synthesis of the methyl group of thymidylic acid, the insertion of the C-2 and C-8 atoms of the purine skeleton, and the β-carbon of serine (Stokstad and Koch 1967).

The biological effects of methotrexate are due entirely to its inhibition of the reductase (Jukes and Broquist 1963). Inhibition of the reductase prevents generation of tetrahydrofolate and ultimately results in a cessation of the reaction requiring folate coenzymes such as biosynthesis of thymidylic acid, purines and serine, and thus the process of DNA synthesis and cellular replication. Borsa and Whitmore (Borsa and Whitmore 1969) suggested that methotrexate may have other sites of action in addition to its inhibition of folate and dihydrofolate reductase. Thymidylate synthetase and, in the presence of excess deoxycytidine, the transformylase enzymes involved in the purine synthesis *de novo* are probably two such sites. Methotrexate can also interfere with the ulitization of existing reduced folates that the competition is not at the transport level.

Since DNA synthesis and cellular replication is important

in rapidly proliferating tissue. Active proliferating tissue such as malignant cells, bone marrow, fetal cells, dermalepithelium, buccal and intestinal mucosa and cells of the urinary bladder are in gereral more sensitive to the effect of methotrexate. Cellular proliferation in malignant tissue is greater than in most normal tissue and thus methotrexate may impair malignant growth more pronouncedly. Hrynuik (Hrynuik 1972) found that methotrexate suppressed incorporation of ^{3}H-thymidine into DNA, ^{3}H-uridine into RNA, of ^{3}H-leucine into protein and killed L5178Y murine lymphoblasts. MTX arrests the growth of tumor cells also by the induction of a "purineless" states in these cells.

Kaminskas and Nussey (1978) found that glycolytic rate of Ehrlich ascites tumor cells decreased with methotrexate treatment, adenylate pools and adenylate energy charge were also decreased in cells isolated in methotrexate containing medium *in vitro*. The purineless state was accompained by a sereve inhibition of ATP regeneration. This suggests that the major cytotoxic effect of methotrexate appears to be due to an energy-depleted state.

Ehrlich ascites tumor cells are highly dependent on glycolysis for their energy metabolism. Other tumor cells and normal cells which depend mainly on respiration for their energy metabolism, may be less sensitive to depletion of adenylate pool and to inhibition of glycolysis by methotrexate (Kaminskas and Nussey 1978). Transformed fibroblasts (Schwartz and Johnson 1976), Novikoff rat hepatoma cells (Plagemann and

Erbe 1973) and reticulocytes (Freudenberg and Mager 1971) tolerate glucose starvation without decreasing their adenylate pool or the adenylate energy charge unless respiration is inhibited as well.

Methotrexate can also inhibit the uptake of 2-deoxy-D-glucose and 3-O-methyl-D-glucose in cultured Ehrlich ascites tumor cells (Kaminskas 1979) but hexokinase activity was found not to be affected. Since the administration of methotrexate can control tumor growth in tumor bearing hosts, it is of value to investigate whether a similar effect of methotrexate on glucose uptake of tumor cells can be obtained when Ehrlich ascites tumor bearing mice are treated with methotrexate *in vivo*.

Furthermore, *in vivo* measurement on the glucose carriers on tumor cells during Ehrlich ascites tumor development and MTX treatment are of interest.

VII. Aim of study

2-deoxy-D-glucose uptake by different days old Ehrlich ascites tumor cells *in vivo* was studied in order to see whether there is any change in glucose uptake by the tumor cells during tumor development. Furthermore, the number of glucose-sensitive cytochalasin B binding site was also measured as a parallel study to confirm the results of the glucose uptake experiments. Since the study on the nature of glucose-sensitive cytochalasin B binding sites on Ehrlich ascites tumor cells are relatively limited, and its relationship to glucose

carrier is not established, therefore the properties of binding specificity of glucose-sensitive cytochalasin B binding sites by using other sugars and glucose uptake inhibitors were studied.

Based on the results of the above experiments, it is suggested that glucose uptake as well as the number of glucose-sensitive cytochalasin B binding site increased progressively during tumor development *in vivo*. It is also shown that the glucose-sensitive cytochalasin B binding sites are closely related if not identical to the glucose carriers which are responsible for the transport of glucose in Ehrlich ascites tumor cells. Therefore the possible factors those may cause the changes in the number of glucose carrier in Ehrlich ascites tumor cells during tumor development are also investigated.

Tumor bearing patients are always hypoglycaemic. Similarly, the blood level of animals carrying various tumors have lower value than normal animals (Del Monte and Rossi 1963; Tagi-Zade and Shapot 1971). In Ehrlich ascites tumor bearing mice, hypoglycemia can also be observed (Pavelic *et al* 1979). On autopsy of the animals from the experimental group, a direct correlation was found between the size of the tumor and the magnitude of the decrease in the blood sugar levels, as well as an inverse correlation between this decrement and the amount of necrose. It appeared that the extent of hypoglycemia depended on the number of viable cancer cells. A similar correlation between the magnitude of hypoglycemia and growth of lymphetic leukemic tumor in mice was observed (Silverstein

et al 1964).

The glucose level in normal mouse peritoneal fluid and cell free peritoneal fluid are 95 and 82 mg/100 ml respectively (Nakamura and Hosoda 1968) which are just slightly lower than that of normal mice serum glucose concentration (Mallick *et al* 1968). By contrast, the ascites fluid as well as the cell free ascites fluid did not contain any detectable glucose (Chan *et al* 1983). Ehrlich ascites tumor cells also do not contain detectable intracellular glucose (Nakamura and Hosoda 1968). Relationship between changes of serum glucose and glucose carrier on tumor cells are also studied.

Since glucose is the major source of energy in Ehrlich ascites tumor cells (Lazo 1981) and it is obvious that glucose uptake is important for the growth of Ehrlich ascites tumor cells. Amos and co-workers (Martineau *et al* 1972) have showed that the rate of glucose (or 2-deoxy-D-glucose) uptake by chick fibroblast cultures is governed by the glucose concentration in the medium during growth. High glucose concentration during growth seems to "repress" glucose transport, whereas low glucose concentration elicits a "derepression" effect on this transport system. In addition, glucose starvation can stimulate the uptake rate of glucose in chick fibroblasts (Shaw and Amos 1973).

Tumor development can cause a significant drop in the elevated level of blood glucose and disappearance of glucosuria in diabetic patients (Vaissman *et at* 1964). In alloxan-induced diabetic rats, Novikoff hepatoma and Walker carcinoma-

256 development were found to reduce the blood sugar level (Goranson *et al* 1954; Goranson and Tilser 1955). In alloxan-induced diabetic mice Ehrlich ascites tumor development can also decrease the blood glucose level (Pavelic *et al* 1979).

Based on the above evidences, it is reasonable to suspect that the decrease in serum glucose of tumor bearing hosts may be a stimulant of increased glucose uptake rate as well as increased number of glucose carriers in Ehrlich ascites tumor cells.

In order to investigate the effect of serum glucose of Ehrlich ascites tumor bearing mice on the number of glucose carriers in tumor cells, carrier number in the tumor cells harvested from mice induced hyperglycemia and hypoglycemia by injecting streptozotocin (Maclaren *et al* 1980; Bond 1980) and insulin (Pavelic 1979) respectively are studied.

However it was found that insulin can increase the rate of glucose uptake in chick embryo fibroblasts and Hela cells (Shaw and Amos 1973), and the translocation of glucose carriers from microsomal storage pool to plasma membrane in rat adipocytes (Suzuki and Kono 1980; Karnceli *et al* 1981; Kono *et al* 1981). In order to clarify the hormonal effects on the number of glucose carriers in Ehrlich ascites tumor cells, glucose carrier number in the tumor cells after the direct incubation with insulin are studied. Furthermore, hypoglycemia developed in Ehrlich ascites tumor bearing mice may induce hyperplasia of the adrenal cortex and hypersecretion of glucocorticoids. The final phase of tumor development was

reported to be characterized by emaciation of the gland, and depletion of the glucocorticoids reserve (Lichter and Sirett 1968; Kawai *et al* 1969). Therefore, the direct effect of the hormones on the number of glucose carriers in Ehrlich ascites tumor cells is of interest to be investigated.

On the other hand, the effect of glucose concentration on glucose carriers are also studied by using *in vitro* incubation. 7 day-old Ehrlich ascites tumor cells were incubated in two different glucose concentration and the change in glucose carriers during incubation were recorded. The result of this experiment can serve as an additional evidence to further confirm the relationship between glucose level and the number of glucose carriers.

As MTX can decrease the growth of many tumor cell lines including Ehrlich ascites tumor cells, and the uptake of 2-deoxy-D-glucose by Ehrlich ascites tumor cells is also inhibited by MTX *in vitro* (Kaminskas 1979). It is of interest to study whether there is a similar effect of MTX on glucose uptake by Ehrlich ascites tumor *in vivo*. Therefore in the final part of the studies, effect of MTX on glucose uptake by the tumor cells is examined. Its effect on the number of glucose carrier in the tumor cells is also studied as a parallel study to the above mentioned experiment.

MATERIALS AND METHODS

Materials

The suppliers of the materials are shown in table:

Table: Materials and Suppliers

Materials	Suppliers
Antibiotics - antimycotics (penicillin 10,000 U/ml fungizone 25 mcg/ml streptomycin 10,000 mcg/ml)	Gibco (Grand Island Biological Company)
D-(-) Arabinose	British Drug House
Calf Serum	Gibco
Corticosterone	E. Merck
Cortisone	E. Merck
Cytochalasin B	Sigma
^{3}H-cytochalasin B (7.2 Ci/mmol)	New England Nuclear
2-Deoxy-D-Glucose	Sigma
2-Deoxy-D-^{3}H-glucose (37.3 Ci/mmol)	New England Nuclear
Diethylstilbestrol	Sigma
D-(-) Fructose	British Drug House
L-(-) Fucose	Sigma
D-(+) Galactose	British Drug House
Glucagon	Sigma
Glucosamine	E. Merck

D-(-) Glucose	British Drug House
L-Glucose	Sigma
Glucose Kit (Kit 510-DA)	Sigma
Hepes	Sigma
Insulin	Sigma
Lactose	British Drug House
Maltose	Ajax
D-(+) Mannose	British Drug House
Methotrexate	Lederle
3-O-methyl-D-glucose	Sigma
Phloretin	Sigma
Protamine zinc insulin	Weddle Pharmaceuticals Ltd. (U.K.)
RPMI 1640	KC Biologocal Inc.
Streptozotocin	Sigma
Sucrose	Mallinckrodt

Methods

I. Maintenance of Ehrlich ascites tumor cell line

Ehrlich ascites tumor, Ny Klein cell type, was maintained by intraperitoneally implantation in albino mice (ICR strain). Mice bearing 7 day-old tumors were killed by cervical dislocation. The pooled tumor cells were washed five or more times with half isotonic saline to remove the blood cells completedly (Wu and Racker 1959) and harvested by centrifugation at 800xg for 90 seconds at room temperature in an IEC table-top clinical centrifuge. The packed cells were then resuspended in phosphate-

buffered saline (PBS), pH 7.4. Mice weighing 30-35 gm were inoculated intraperitoneally with 10^7 washed Ehrlich ascites tumor cells in 0.2 ml PBS. Cell count was determined with a haemocytometer.

II. Uptake of 2-deoxy-D-glucose by Ehrlich ascites tumor cells

To determine the rate of 2-deoxy-D-glucose uptake by Ehrlich ascites cells, the procedure of Kaminskas (Kaminskas 1979) was used with minor modification. Mice were inoculated with Ehrlich ascites tumor cells on day 0. Tumor bearing mice were killed on days 2, 4, 5, 7, 9 and 11. The harvested tumor cells were pooled and washed free of blood cells and finally resuspended in PBS enriched with 3.2 mM $CaCl_2$, 1.5 mM $MgSO_4$, 6.1 mM pyruvate, 6.7 mM fumate and 6.1 mM glutamate, pH 7.4 (incubation buffer). Tumor cells from the group of day 2 were harvested from 40 mice while those of day 4 were harvested from 20 mice. Cell concentration was adjusted to 10^8/ml in incubation buffer. Aliquots (1 ml) of cell suspension were placed in stoppered flasks and equilibrated to 37° C in a shaking water bath for 15 minutes. One ml of 2-deoxy-D-^{3}H-glucose (1μCi/umol) was then added to give final concentrations of 0.25, 0.33, 0.50, 1.00 and 2.50 mM. The content of the flasks were rapidly mixed and the incubation continued at 37° C. Samples of 200 μl were removed after 15, 35, 60, 90, 120, 150 and 180 seconds and added with rapid mixing to test-tubes containing 3 ml of ice-cold PBS supplemented with 15 mM 2-deoxy-D-glucose. Cells were collected by centrifugation at 800xg for 90 seconds at 4°C in a

Beckman J2-21 refrigerated centrifuge. The supernatant was removed by aspiration and the packed cells were washed once again with 3 ml of the above buffer. Cell lysis was accomplished by adding 2 ml of distilled water and mixing with Vortex mixer. One ml of lysate were then mixed with 7 ml of a Triton X-100 - toluene scintillant (Patterson and Greene 1965) and counted in a Beckman LS 7000 liquid scintillation counter.

The uptake of 2-deoxy-D-glucose were calculated from counts per minute of the samples against a standard curve. The inital rate of uptake (Vo) of 2-deoxy-D-glucose at each sugar concentration used was measured graphically. The kinetic parameters of the uptake process, namely, the maximal uptake rate (Vmax) and apparently half-saturation constant (K_M) were determinated by correlating Vo with sugar concentration according to the double-reciprocal formulation of Lineweaver and Burk after correction for non-specific diffusion (unsaturable diffusion) (Renner, Plagemann and Bernlohr 1972; Plagemann and Richey 1974).

III. Glucose-sensitive cytochalasin B binding sites on Ehrlich ascites tumor cells

Equilibrium binding of cytochalasin B to Ehrlich ascites tumor cells were performed according to the methods which were commonly used for the measurement of the glucose carriers on red blood cells (Jung and Rampal 1977; Pinkofsky *et al* 1978; Cuppoletti, Mayhew and Jung 1981) with slight modifications. Ehrlich ascites tumor cells were obtained and resuspended in

PBS as described. Cell concentration were adjusted to 2 x 10^7 / ml. For measuring the total cytochalasin B binding 1 ml of incubation mixture contained 10^7 cells, 0.02 µCi ^{3}H-cytochalasin B and 2×10^{-9} - 5×10^{-6} M cytochalasin B. To assess the glucose-insensitive binding, D-glucose was also added to the incubation mixture to a final concentration of 500 mM. The mixtures were allowed to equilibrate for 20 minutes at room temperature in a Thermolyne speci-mix (Thermolyne U.S.A.). Bound ligand was separated from free ligand by centrifugation at 25,000xg for 20 minutes at 4° C in a Beckman J2-21 refrigerated centrifuge (Beckman U.S.A.). An aliquot (0.5 ml) of the supernatant was removed and its radioactivity was counted. The test-tubes were then drained and the residual supernatant was carefully decanted and wiped with cotton swabs. 1 ml of distilled water was added to lyse the tumor cells, after which 0.5 ml of the cell lysate was used for radioactivity measurement.

The cytochalasin B bound was calculated as percentage of total. The difference in cytochalasin B binding between in the absence (total binding) and presence (glucose-insensitive binding) of 500 mM D-glusoce is the glucose-sensitive binding of cytochalasin B on Ehrlich ascites tumor cells.

Inasmuch as the glucose-sensitive cytochalasin B binding sites have been shown to represent the functional glucose carriers in various cell types studied (Karnieli *et al* 1981; Coppoletti, Mayhew and Jung 1981; Sogin and Hinkle 1980 a; Suzuki and Kono 1980; Jung and Rampal 1977; Lin, Santi and Spudich

1974; Pinkofsky *et al* 1978), the putative glucose carriers on Ehrlich ascites tumor cells were determined by Scarchard analysis of the glucose-sensitive cytochalasin B binding on the tumor cells.

IV. Glucose level in the serum and ascitic fluid of tumor bearing mice

Glucose level in the sera and ascites fluid of tumor bearing and normal mice were determined by using Glucose Kit supplied from Sigma Chemical Company (Kit 510-DA). Blood samples were obtained from mice after overnight fasting. Cell-free ascites fluid was obtained by centrifugation the peritoneal exudate from tumor bearing mice at 800xg for 5 minutes. Protein of the samples (ascites fluid and sera) were removed with the addition of half volume of 0.3 N barium hydroxide and zinc sulfate. 5.0 ml combined enzyme-color reagent (glucose oxidase and o-dianisidine dihydrochloride) solution were added to deproteinized samples. All samples were incubated at 37° C for 30 minutes and then the absorbance of the samples at 440 nm was determined. The glucose level of the samples were calculated from their absorbance at 440 nm against glucose standard curve.

V. Effect of methotrexate (MTX) on glucose uptake and glucose carrier on Ehrlich ascites tumor cells

Mice were inoculated with 10^7 ascites tumor cells harvested from 7 day-old tumor in 0.2 ml PBS on day 0 as described. The effect of MTX was examined in groups of at least ten mice each.

In the test groups, MTX (0.4 mg/Kg body weight) in normal saline was administered on days 2, 4 and 6 (group A) or on days 4, 5 and 6 (group B) post implantation. In the control group, only saline was injected. On day 7, all groups of mice were killed and the tumor cells were harvested and washed. The glucose uptake and glucose carriers on Ehrlich ascites tumor cells were then determined.

VI. Specificity of the glucose carriers on Ehrlich ascites tumor cells

In these experiments, 7 day-old tumor cells were used. Equilibrium binding of cytochalasin B was performed in the presence and absence of 500 mM of D-(+) mannose, D-(+) galactose, D-(-) fructose, L-glucose, 3-O-methyl-D-glucose, 2-deoxy-D-glucose, D-(-) arabinose, glucosamine, L-(-) fucose, maltose, lactose and sucrose; and two glucose uptake inhibitors, phloretin and diethylstilbestrol, at the concentration of 5×10^{-5} M and 1×10^{-4} M respectively. Binding data were analysed by Scathard plots as for D-(+)-glucose.

VII. Effects of diabetes on glucose carriers on Ehrlich ascites tumor cells *in vivo*

Tumor bearing mice weighing 30-35 gm were devided into four groups with 10 mice in each group: insulin treated, streptozotocin-induced diabetic, diabetes with insulin treatment and control groups.

In insulin treated group, 4 IU/mouse of insulin were subcutaneously injected into the mice 18 hours before inoculation

of 10^7 tumor cells. Insulin was then injected every 36 hours post implantation. The dosage of insulin was 4 IU/mouse in the first and second injection, 3 IU/mouse in the third injection, 2 IU/mouse in the fourth injection, and 1 IU/mouse in the fifth injection.

In streptozotocin-induced diabetic group, streptozotocin (200 mg/Kg body weight) in citrate buffer, pH 4.2 was intraperitoneally injected into the mice three days before the inoculation of tumor cells. Blood glucose of streptozotocin treated mice was determined prior to the inoculation of 10^7 tumor cells to ensure the diabetogenic effect of streptozotocin (Junod *et al* 1967; Brosky and Logothetpoulos 1969; Renold *et al* 1974).

In the group of diabetes with insulin treatment, the mice were induced diabetic with streptozotocin as described above. Blood glucose was determined prior to the injection of insulin. The inoculation of 10^7 tumor cells was performed 18 hours after the first injection of insulin. The dosage and intervals of insulin injections were same as described in the insulin treated group.

In the control group, only normal saline was injected into the Ehrlich ascites tumor bearing mice inoculated with 10^7 cells.

Serum glucose of the four groups of mice was determined every two days during the development of tumor cells. Blood samples were obtained after 4 hours of fasting of the mice.

All groups of mice were killed seven days after tumor inoculation by cervical dislocation. Tumor cells were harvested,

washed and the glucose carriers on the tumor cells were determined by methods as described above.

VIII. Hormonal effect on glucose carriers on Ehrlich ascites tumor cells *in vitro*

Tumor cells were harvested from mice bearing 7 day-old tumors. After the removal of the blood cells by half-isotonic saline, tumor cells were resuspended in PBS supplemented with 3.2 mM $CaCl_2$, 1.5 nM $MgSO_4$, 6.1 mM pyruvate, 6.7 mM fumarate, 6.1 mM glutmate and 0.56 mM glucose. Different hormones were added to the cell suspension to examine their effects on the number of glucose carriers in cells. The tumor cells were incubated at 37^{o} C for 60 minutes in the presence or absence of different hormones. Cell concentration in incubation buffer is 2×10^{7}/ml. The concentration of insulin were 0.5 IU/ml and 0.05 IU/ml designated as high and low dosage of insulin respectively, that of glucagon was 5×10^{-3} mg/ml and those of corticosterone and cortisone were 25×10^{-3} mg/ml. These hormonal concentrations are essentially comparable with those in the study of hormonal effects on the glucose uptake by erythrocytes, chick fibroblasts and Hela cells (Lacko *et al* 1975; Shaw and Amos 1973; Lacko, Wittle and Lacko 1977). After 60 minutes of incubation, the tumor cells were harvested by centrifugation in an IEC table-top clinical centrifuge and washed twice with PBS at room temperature. Then the washed cells were assayed for glucose carriers.

IX. Effect of glucose concentration on glucose carriers on Ehrlich ascites tumor cells *in vitro*

Tumor cells were harvested from mice bearing 7 day-old tumors. After the removal of the blood cells by half-isotonic saline, tumor cells were resuspended in RPMI 1640 medium supplemented with 2 mM L-glutamine, 5% heat-inactivated calf serum, 3.5% cell free ascites fluid, antibiotics-antimycotics mixture giving a final concentration of penicillin to 100 U/ml, fungizone to 0.25 mcg/ml and streptomycin to 100 mcg/ml. Glucose was then added to give final concentrations of 1.9 and 5.5 mg/ml. The cell suspensions were mixed with glucose and incubated at 37^{o} C. The cell concentration was adjusted to 4×10^{6}/ml. Aliquots of cell suspension were taken out in different time intervals. Cells were harvested by centrifugation and washed twice with PBS at room temperature. The washed tumor cells were then assayed for glucose carriers. The supernatant in the aliquots were determined for glucose concentration.

RESULTS

I. Growth characteristics of Ehrlich ascites tumor cells in mice

Before experiments with Ehrlich ascites tumor cells were performed, the growth characteristics of Ehrlich ascites tumor cells *in vivo* have been studied. Mice were inoculated intraperitoneally with 10^7 Ehrlich ascites tumor cells as described in Materials and Methods on day 0. Fig. 1A shows the growth curve of Ehrlich ascites tumor cells *in vivo*. In the first two days post implantation, the tumor cells grew slowly. This period is designated as "latency period" as originally suggested by Klein and Revesz (1953). Between day 2 and day 7, tumor cells grew most rapidly while in the period of day 7 to day 11, tumor cell number increased slowly and seemed to be level off. This last part of the growth curve is designated as "terminal phase" (Klein and Revesz 1953). Although the cell number increased slowly in terminal phase, the volume of ascites fluid increased tremendously in this phase (Fig. 1B) leading to a slight decrease in cell concentration of tumor cell in ascites fluid (Fig. 1C).

Fig. 2 shows the change in net body weight after exhaustive drainage of tumor cells during tumor development. Net body weight of tumor bearing mice increases significantly comparing to that of day 0 during tumor development and reaches a peak value of 36.8 gm on day 4 ($p < 0.05$) and then decreases significantly ($p < 0.01$) afterwards. The decrease in net body weight of tumor bearing mice may be due to cancer cachexia which is

Fig. 1 Growth characteristics of Ehrlich ascites tumor bearing mice during tumour development. Tumor bearing mice were inoculated with 10^7 cells on day 0. Total tumor cell number, volume of ascites fluid and cell concentration in ascites fluid were determined on indicated days post transplanatation. Values are presented as mean ± S.E.M. for three experimental groups.

A. Growth curve (semi-log) of Ehrlich asictes tumor cells in mice. Cells were harvested from mice and counted with haemocytometer.

B. Volume of total ascites fluid. Ascites fluid was drained from tumor bearing mice and mueasured.

C. Tumor cell concentration in ascites fluid.

FIGURE 1A

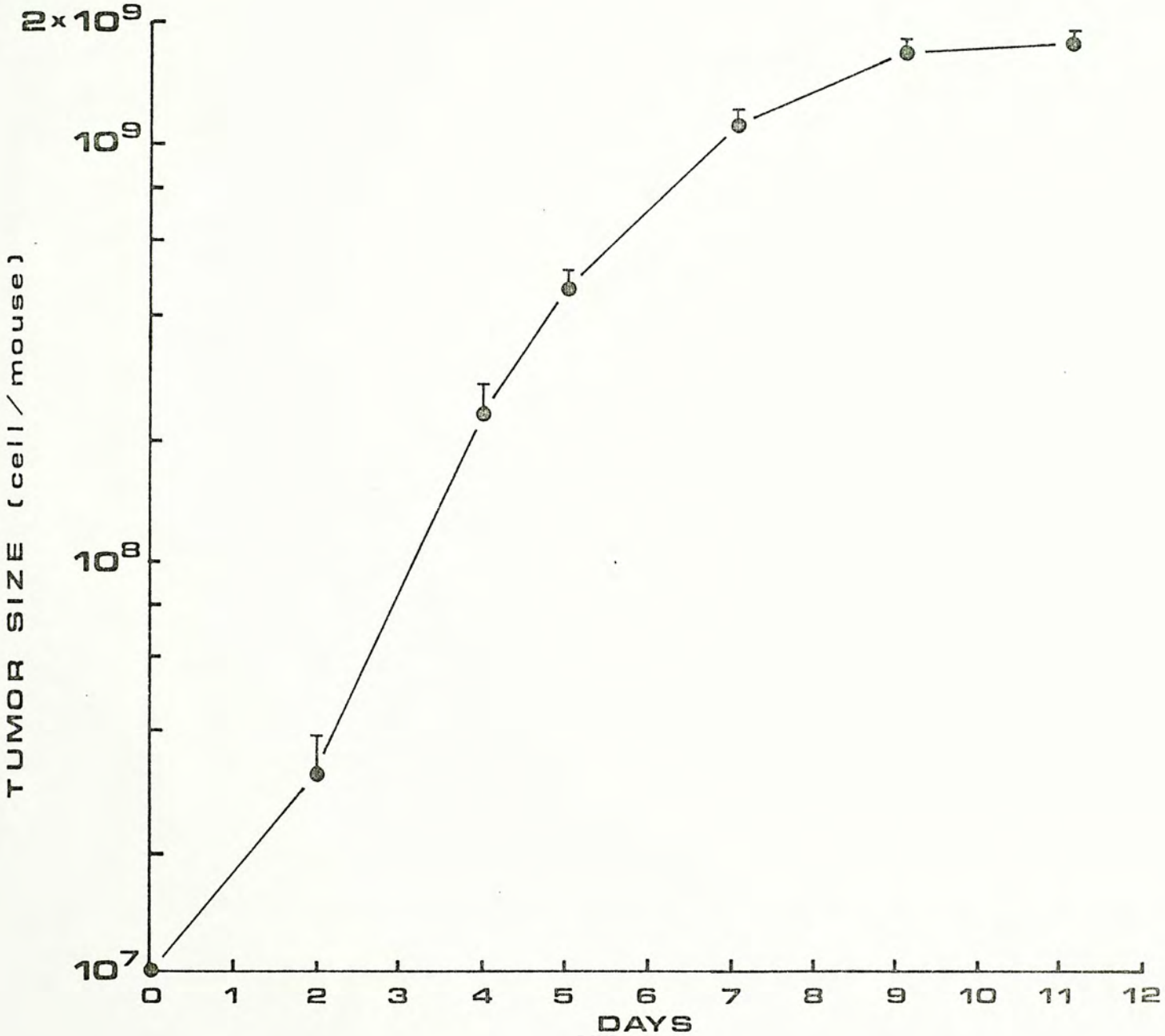

2×10⁹
10⁹
10⁸
10⁷
TUMOR SIZE (cell/mouse)
0
1
2
3
4
5
6
7
8
9
10
11
12
DAYS

FIGURE 1B

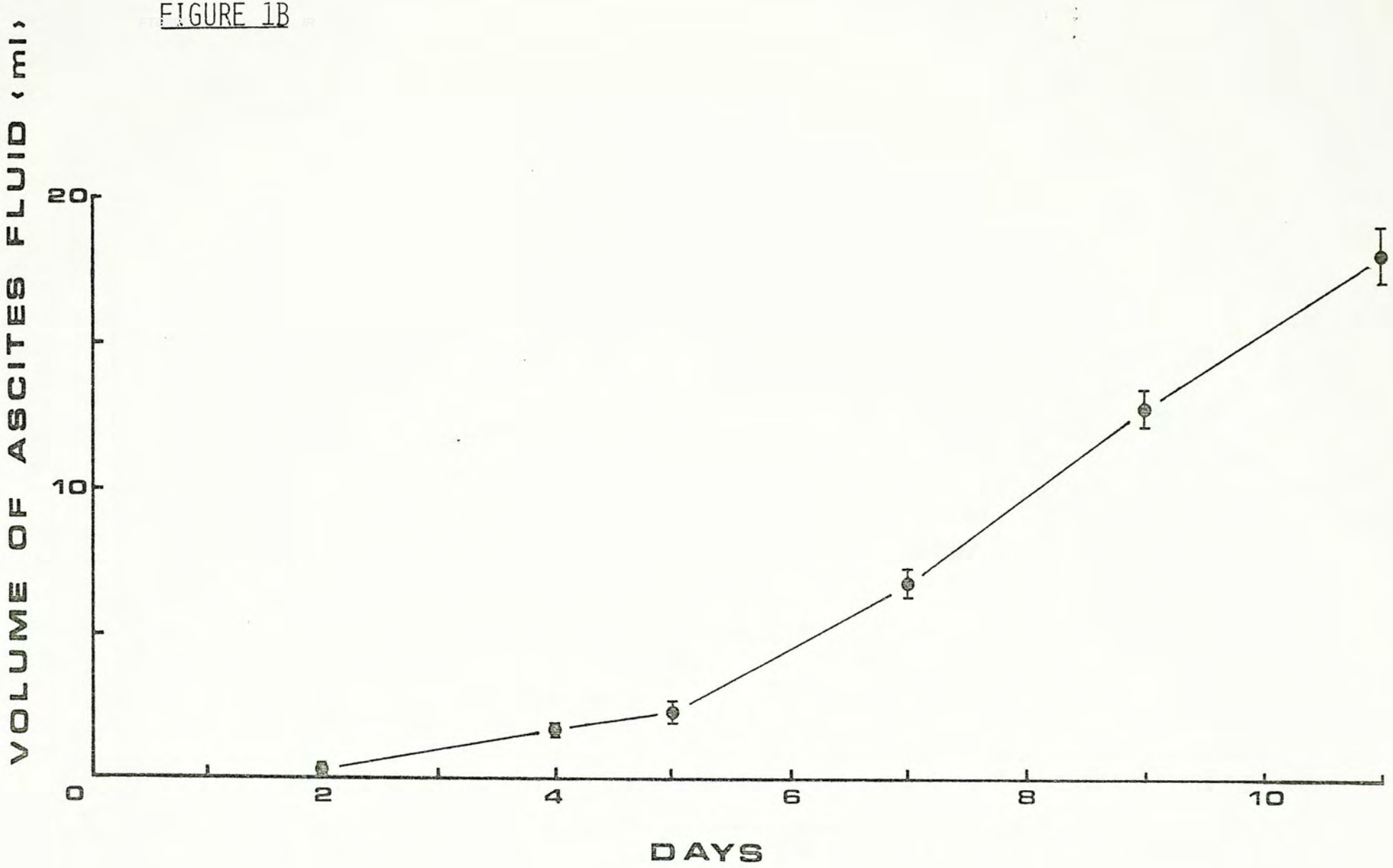


FIGURE 1C

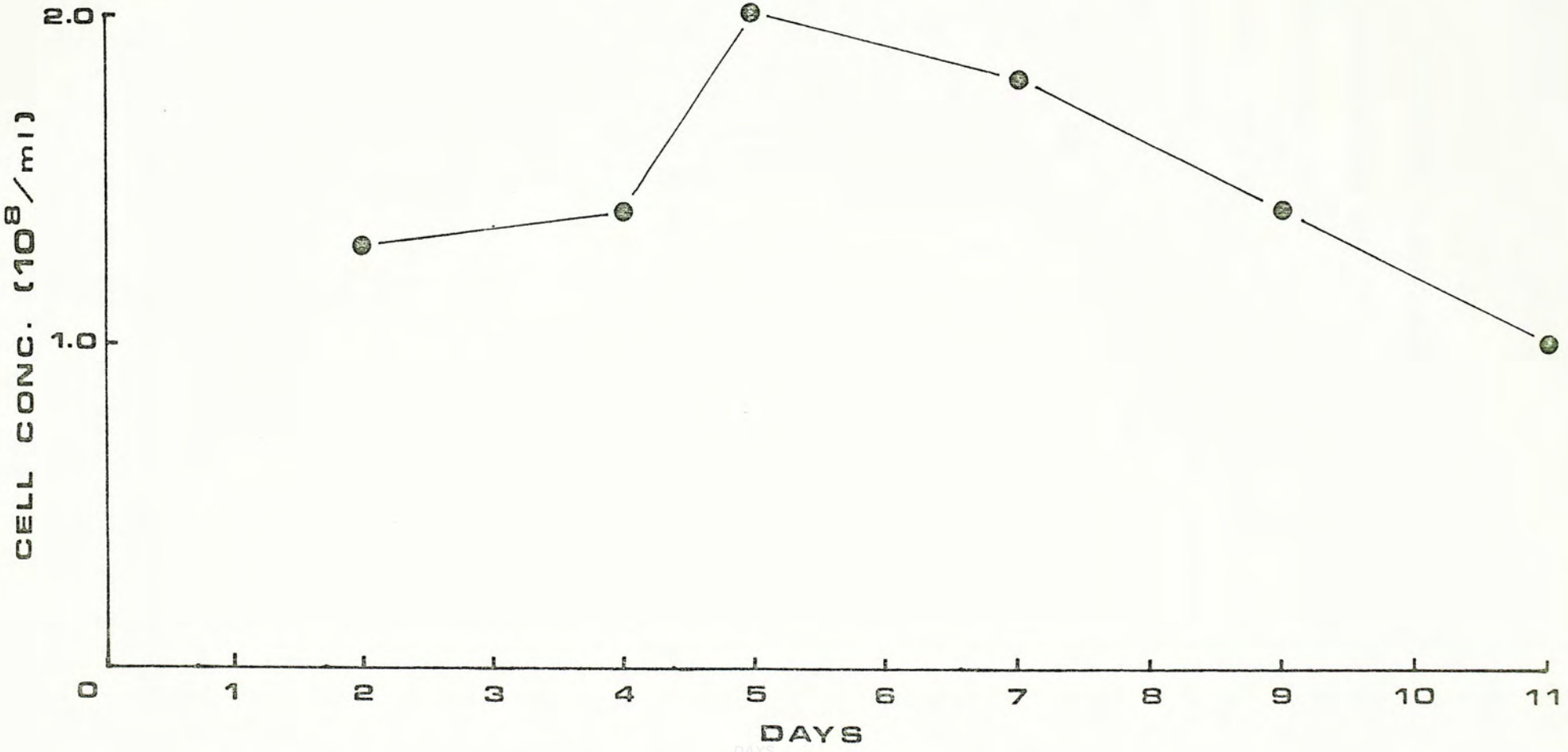
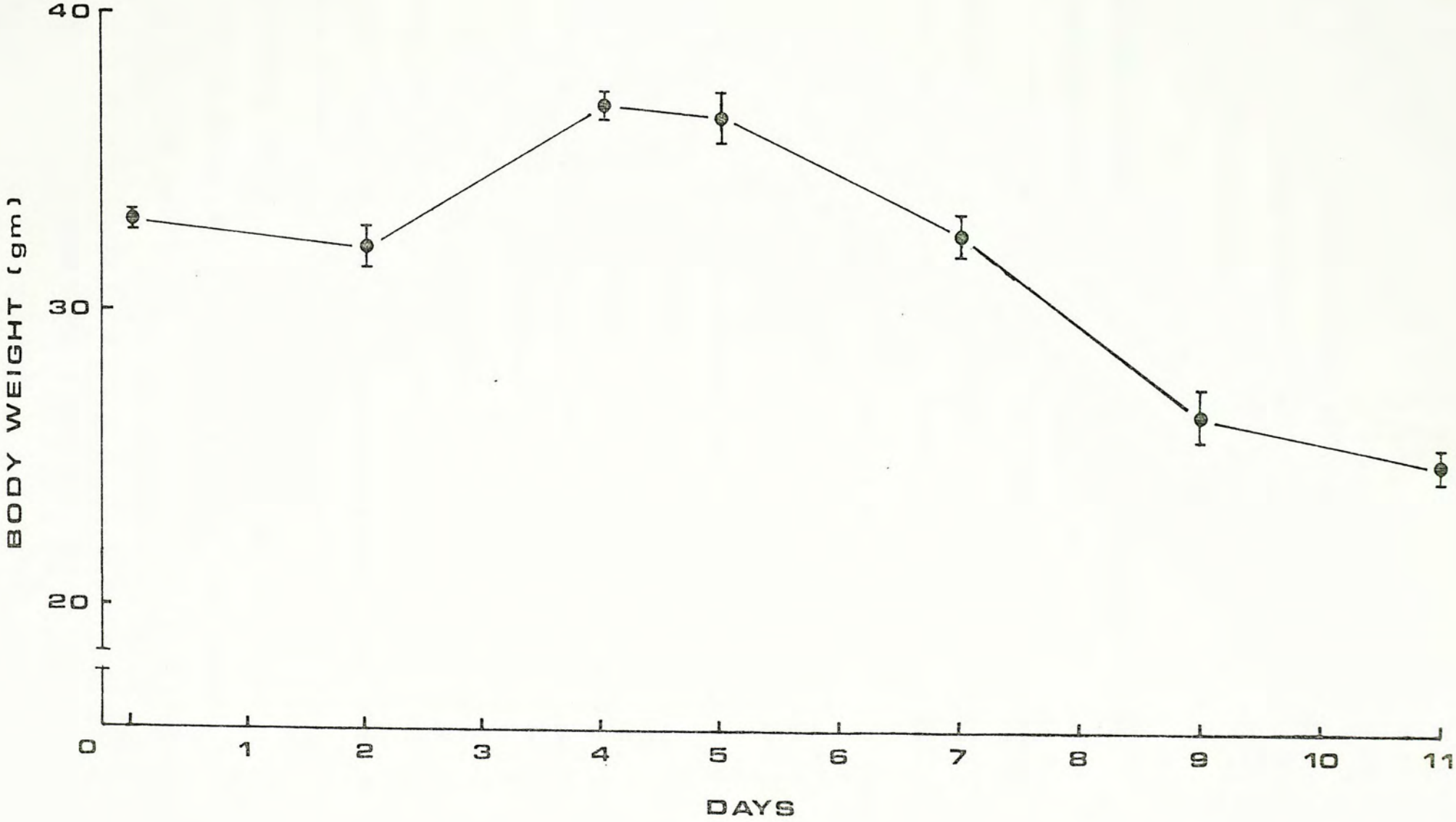


Fig. 2 Changes in net body weight of Ehrlich ascites tumor bearing mice. Mice were inoculated with 10^7 tumor cells on day 0. Mice were weighed after the exhaustive drainage of tumor cells and ascites fluid from their peritoneal cavities on indicated days post transplantation. Values are presented as mean ± S.E.M. for three experimental groups.

FIGURE 2



commonly found in tumor bearing animals (Shapot 1972), however, the reason of the increase in net body weight of tumor bearing mice in the early period is not understood.

II. Changes in 2-deoxy-D-glucose uptake rate of Ehrlich ascites tumor cells during tumor development *in vivo*

Uptake of 2-deoxy-D-glucose by Ehrlich ascites tumor cells during tumor development was studied. Fig. 3A shows a typical time curve of incorporation of 2-deoxy-D-^{3}H-glucose when 7 day-old tumor cells were exposed to various concentrarion of 2-deoxy-D-gucose. The rate of uptake was rapid in the first 10 seconds; it then fell gradually with time. When the initial rate was plotted against the concentration of 2-deoxy-D-glucose (Fig. 3B, curve A), considerable deviation from normal Michaelis-Menten kinetics was seen. Similar observation has been made with the transport of 2-deoxy-D-glucose (Renner, Plagemann and Bernlohr 1972) and nucleosides (Plagemann 1971) in Novikoff rat hepatoma cells and it has been suggested that at high enough concentration, 2-deoxy-D-glucose can enter the cells at a significant rate by simple diffusion (unsaturable component). The extent of the diffusion-mediated uptake can be estimated graphically by drawing a line (Fig. 3B, curve B) through the origin and parallel to the linear portion of the uptake curve (Renner, Plagemann and Bernlohr 1972; Plagemann and Richey 1974). The uptake rate due to the transport reaction was obtained by subtracting the estimated diffusion rate due to unsaturable component from the overall rate (Fig. 3B, curve C). The corrected

rates showed that the uptake of 2-deoxy-D-glucose by Ehrlich ascites tumor cells is a saturable process, and follows Michaelis-Menten kinetics. Lineweaver-Burk plot of the facilitated uptake rate (Fig. 3C) of 2-deoxy-D-glucose by 7 day-old Ehrlich ascites tumor cells yielded a typical best-fit straight line by linear regression from which the kinetic parameters, Vmax and K_M, representing respectively the maximal uptake rate and apparent half-saturation constant of the uptake process can be determined.

Fig. 4 shows that the values of Vmax increased linearly with the progress of tumor growth, rising from 66 nmol/min/$5x10^6$ cell on day 2 to 242 nmol/min/$5x10^6$ cell on day 11 post implantation of Ehrlich ascites tumor cells. By contrast the values of K_M remained unchanged throughout the course of the experiment at about 0.6 mM. These observation can be interpreted to indicate that the nature of the hexose carrier molecules appeared to be relatively unaffected, the transport activity of such molecules increased progressively with the tumor development. The increase in transport activity may be due to the increase in the number of the carrier molecules or alternatively, the turnover rate of the glucose transport system may have increased as the tumor grows. To differentiate between these two possibilities, the equilibrium binding of cytochalasin B on Ehrlich ascites tumor cells was studied.

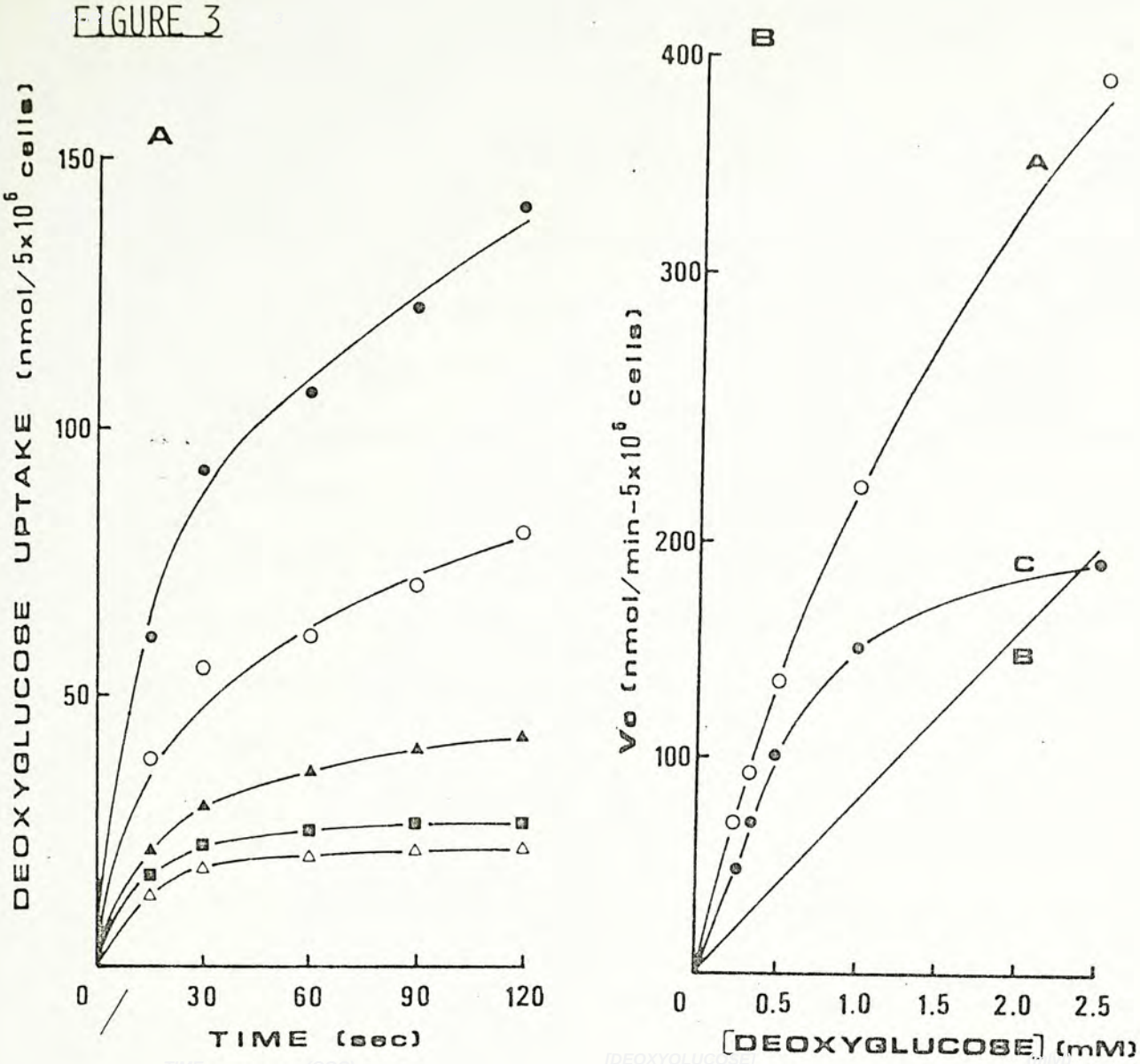
Fig. 3 Representative experiment of the uptake of 2-deoxy-D-glucose by 7 day-old Ehrlich ascites tumor cells.

A. Initial rate of uptake by 7 day-old tumor cells at various final concentrations of 2-deoxy-D-glucose; 0.25 mM (△-△); 0.33 mM (▨-▨); 0.5 mM (▲-▲); 1.0 mM (○-○); 2.5 mM (●-●).

B. Initial rates of 2-deoxy-D-glucose uptake by 7 day-old tumor cells as a function of substrate concentrations. Total uptake, determined graphically from Fig. 3A was used to construct curve A (○-○). The rate of simple diffusion (curve B) was determined by drawing a line through the origin parallel to the linear position of curve A (Renner *et al.*, 1972). The rate of uptake due to facilitated diffusion (●-●) was determined by subtracting these values from the rate of total uptake. It was seen that the facilitated uptake process follows Michaelic-Menten kinetics.

C. The Lineweaver-Burk plot of facilitated diffusion determined in Fig. 3B. The kinetic parameters, Vmax and Km were obtained as 172 nmol/min/5 x 10^6 cell and 0.83 mM respectiveiy.

FIGURE 3


A
DEOXYGLUCOSE UPTAKE (nmol/5x10^6 cells)
150
100
50
0
30
60
90
120
TIME (sec)
B
Vo (nmol/min-5x10^6 cells)
400
300
200
100
A
C
B
0
0.5
1.0
1.5
2.0
2.5
[DEOXYGLUCOSE] (mM)

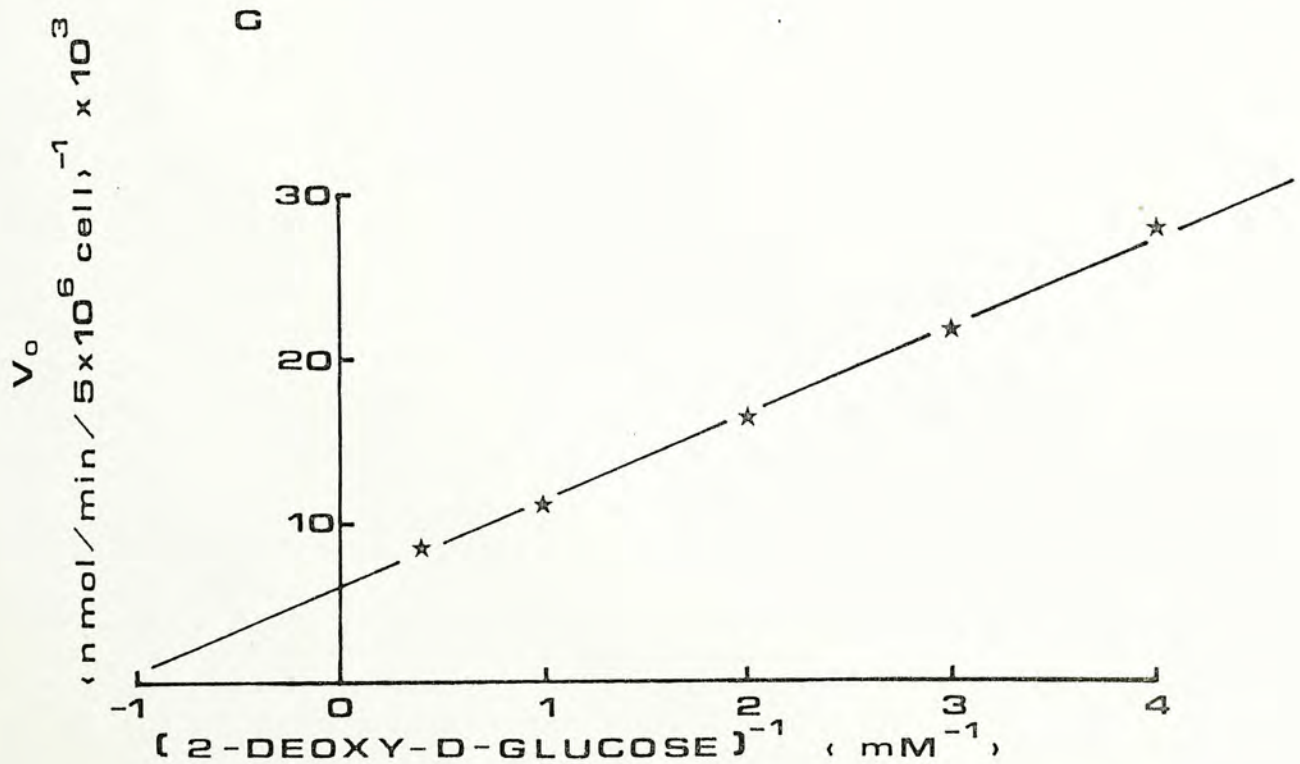
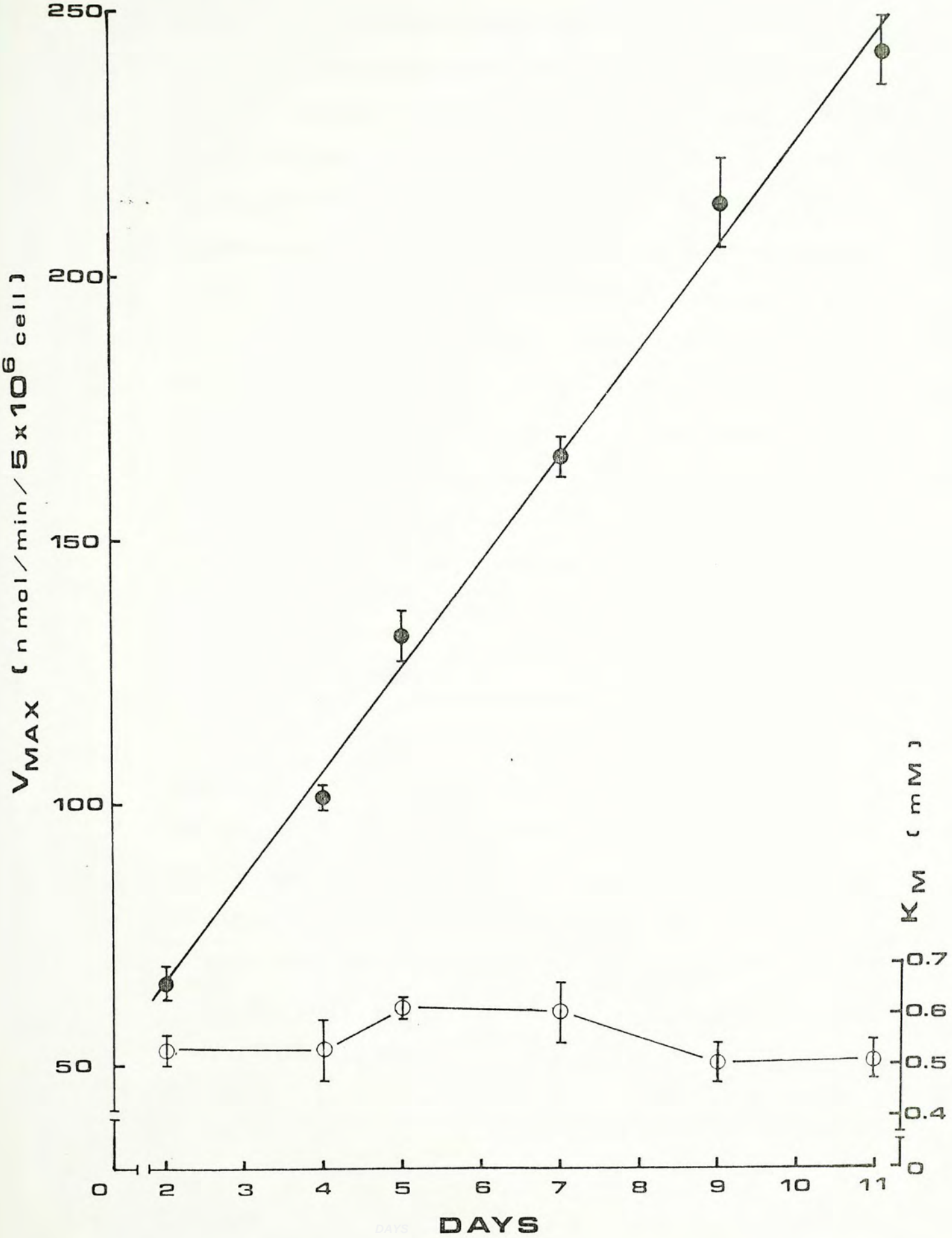

C
Vo
(nmol/min/5x10^6 cell)^-1 x 10^3
30
20
10
-1
0
1
2
3
4
(2-DEOXY-D-GLUCOSE)^-1 (mM^-1)

Fig. 4 Changes in Vmax (●-●) and Km (○-○) for uptake of 2-deoxy-D-glucose during tumor development. Values are presented as mean ± S.E.M. for triplicate determinations in two separate experiments. Statistical analysis for significance of difference of Vmax on different days post transplantation relative to data of day 2 was performed using student t-test. P values of days 4, 5, 7, 9 and 11 for the significance of difference were < 0.001. Km values on different days post transplantation remained relatively unchanged.

FIGURE 4


250
200
150
100
50
0
2
3
4
5
6
7
8
9
10
11
DAYS
V_{MAX} (n mol / min / 5×10^6 cell)
K_M (mM)
0.7
0.6
0.5
0.4
0

III. Glucose-sensitive cytochalasin B binding site on Ehrlich ascites tumor cells during tumor development

Fig. 5A shows the results of a representative experiment using cells from 7 day-old tumors. The presence of 500 mM D-glucose in the incubation medium inhibited the binding of cytochalasin B in a competitive manner. This glucose-sensitive binding site for cytochalasin B, comprising approximately 60% of the total cytochalasin B binding sites, has been identified as the glucose transporter in several cell lines including cultured Ehrlich ascites tumor cells (Pinkofsky, Rampal, Cowden & Jung 1978; Karnieli *et al* 1981; Sogin and Hinkle 1980; Suzuki and Kono 1980; Lin, Santi and Spudick 1974; Cuppoletti, Mayhew and Jung 1981). When the difference in cytochalasin B bound in the presence of 500 mM D-glucose and the absence of D-glucose from Fig. 5A was analysed by the Scatchard plot, a best-fit linear plot was obtained by linear regression (Fig. 5B), from which it was estimated that for 7 day-old tumor cells, the density of glucose-sensitive cytochalasin B binding site (Bo) was 215 pmol/10^7 cell; the dissociation constant (K_d) was 2.7×10^{-7} M. Fig. 6 shows that as the tumor developed the number of glucose-sensitive cytochalasin B binding site (Bo) increased progressively. When compared with the Bo value for 2 day-old tumor cells, the difference was highly significant ($p < 0.001$). The apparent dissociation constant (K_d), however, remained unchanged.

Fig. 5 Representative experiment on determination of glucose-sensitive cytochalasin B binding sites on 7 day-old Ehrlich ascites tumor cells.

A. Representative experiment showing the binding of cytochalasin B by Ehrlich ascites tumor cells harvested from 7 day-old tumors as a function of ligand concentrations. Cytochalasin B bound was calculated as percent of total. Percentages of cytochalasin B bound in the absence (▲ - ▲) and in the presence (● - ●) of 500 mM D-glucose are shown. The difference represents the D-glucose-sensitive portion.

B. Scatchard analysis of the D-glucose-sensitive binding of cytochalasin B to Ehrlich ascites tumor cells determined from Fig. 5A. Control data from Fig. 5A were used to calculate the bound and free ligand. The line was the best fit according to linear least squares analysis. The binding parameters, total binding site (Bo) and dissociation constant (Kd) for D-glucose-sensitive cytochalasin B binding site on Ehrlich ascites tumor cell were determined to be 215 pmol/10^7 cells and 2.4 x 10^{-7} M respectively.

FIGURE 5

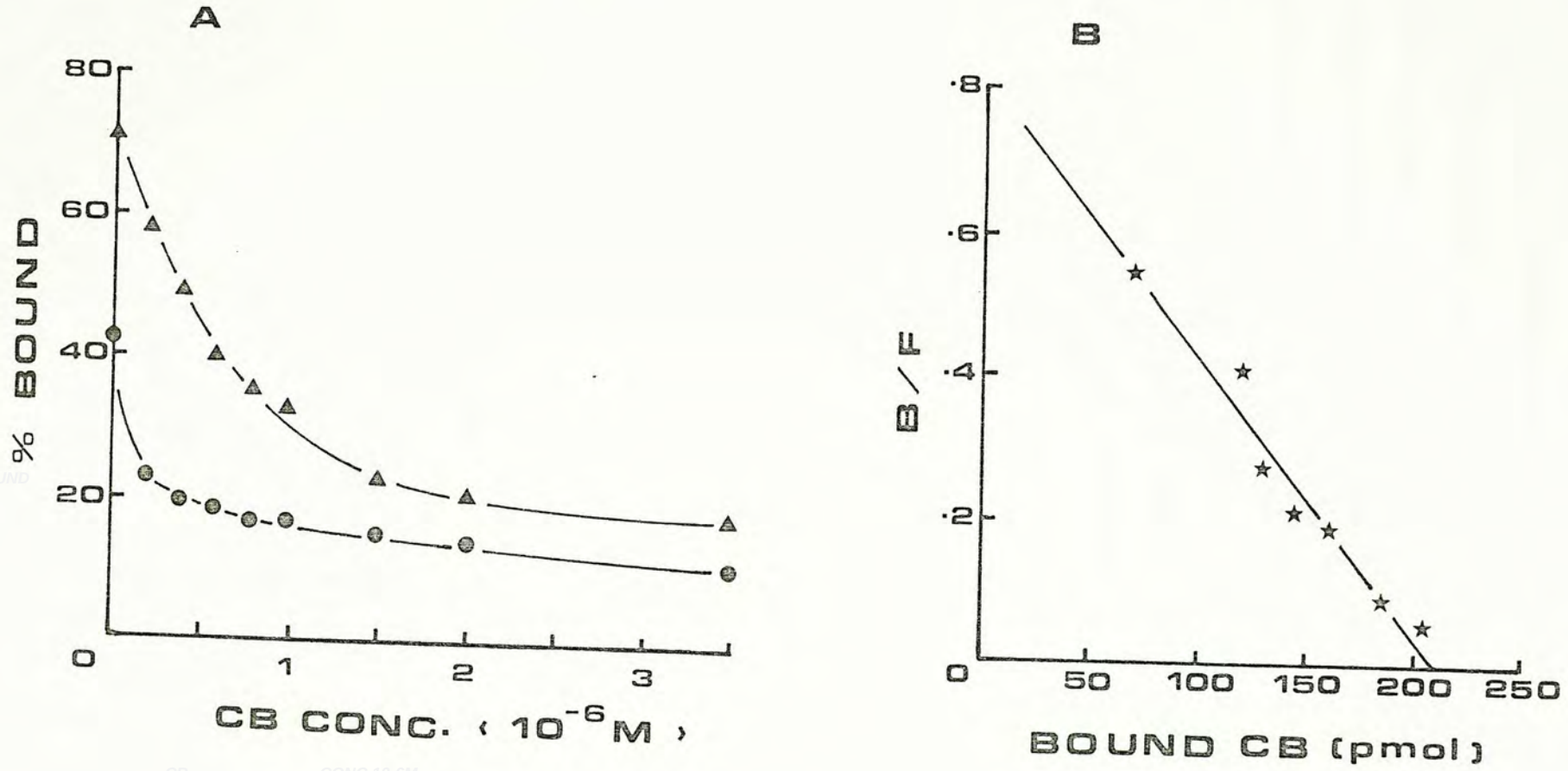
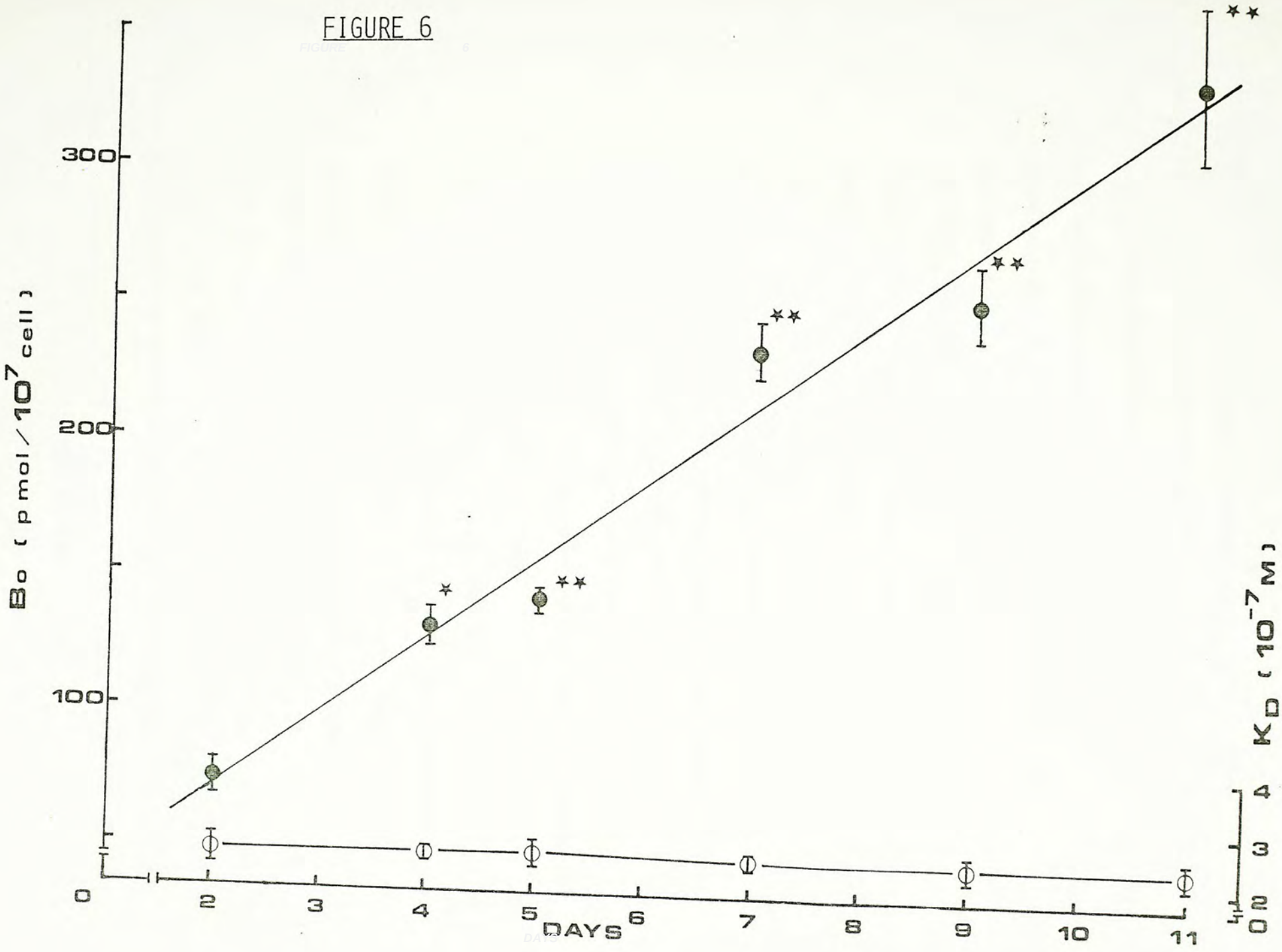

A
80
60
40
20
0
% BOUND
1
2
3
CB CONC. ‹ 10⁻⁶M ›
B
·8
·6
·4
·2
0
B/F
50
100
150
200
250
BOUND CB (pmol)

Fig. 6 Changes in Bo (●-●) and Kd (○-○) values for D-glucose-sensitive binding of cytochalasin B during tumor development. Values are presented as mean ± S.E.M. for three separate experiments. Statistical analysis for significance of difference of Bo on different days post transplantation relative to day 2 value was performed using the Student t-test. ★, P value for the significance of different < 0.01; ★★, P value for the significance of difference < 0.001. The Kd values remained relatively unchanged.

FIGURE 6



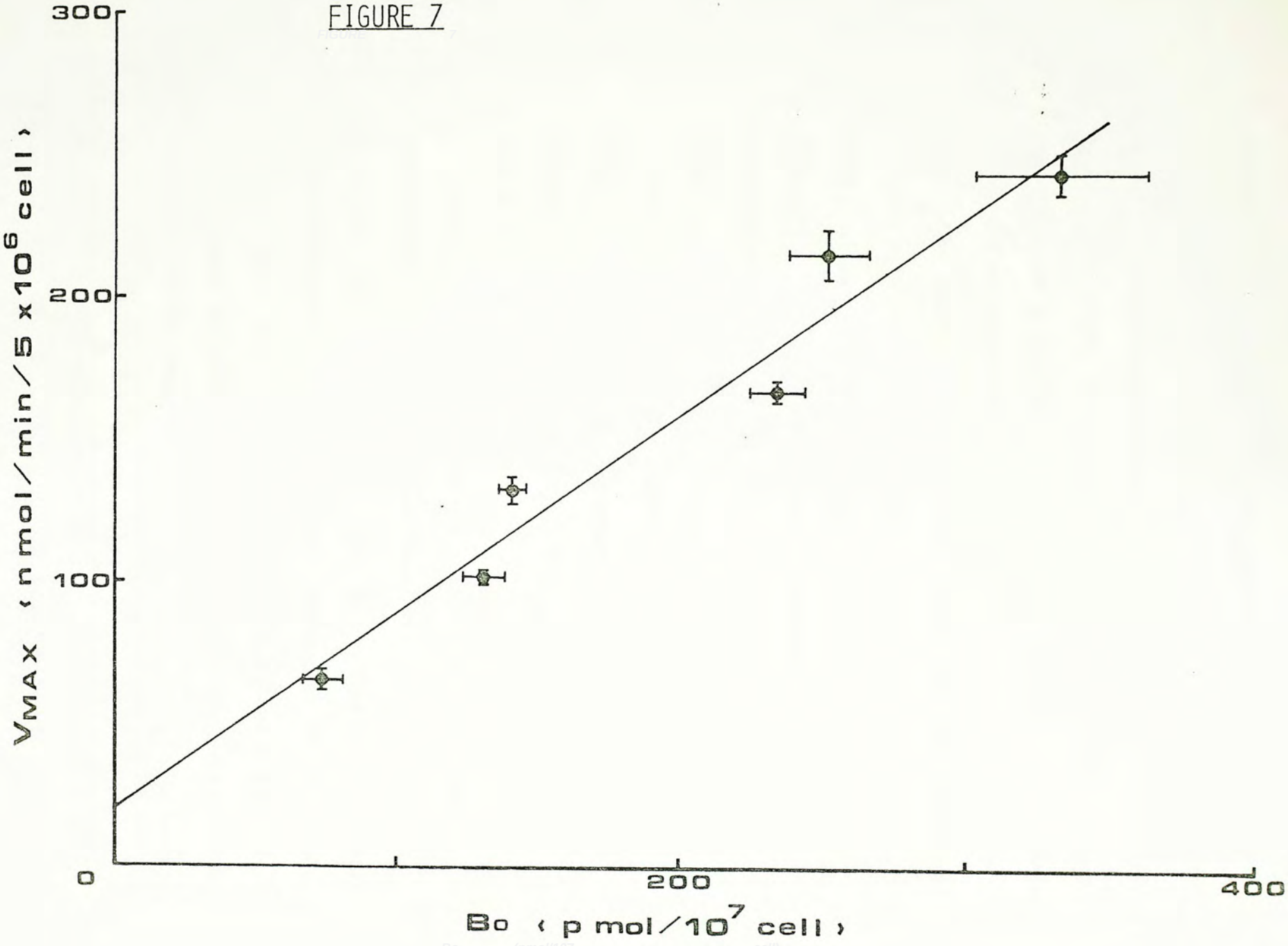
IV. Correlation between glucose uptake rate and glucose-sensitive cytochalasin B binding site on Ehrlich ascites tumor cells during tumor development

Since both glucose uptake rate and glucose-sensitive cytochalasin B binding site increased progressively during tumor development (Fig. 4 and Fig. 6), it is of value to find out the correlation between them. Fig. 7 shows a plot of maximal glucose uptake rate (Vmax) versus the number of glucose-sensitive cytochalasin B binding site (Bo) in the Ehrlich ascites tumor cells on different days post implantation during tumor development in mice. A best-fit straight line was obtained by linear regression with the correlation coefficient greater than 0.976. From this result, glucose-sensitive cytochalasin B binding site is proven to be a good index in representing the glucose uptake activity of the tumor cells. Therefore in the following experiments, only the determination on the number of glucose-sensitive cytochalasin B binding site were studied.

In addition to the findings of the researches working on the glucose transporter in various cell lines including Ehrlich ascites tumor cell (Pinkofsky *et al* 1978; Karbieli *et al* 1981; Sogin and Hinkle 1980; Suzuki and Kono 1980; Lin, Santi and Spudick 1974; Cuppoletti, Mayhew and Jung 1981), our results also confirm the fact that glucose-sensitive cytochalasin B binding site is very closely related, if not identical to the glucose carrier of the cell lines. The increase in number of glucose carrier sufficiently accounted for the elevated rate of glucose uptake associated with tumor development.

Fig. 7 A plot of glucose uptake rate (Vmax) and the corresponding D-glucose-sensitive cytochalasin B binding site (Bo) on Ehrlich ascites tumor cells on indicated days during tumor development. Values are presented as mean ± S.E.M. for three experiments. The line was the best fit according to linear least squares analysis with the correlation coefficient greater than 0.976.

FIGURE 7



V. Specificity of glucose carrier on Ehrlich ascites tumor cells

Since a final concentration of 500 mM D-glucose was added to the incubation mixture to displace the cytochalasin B bound on the tumor cells, it is of value to investigate whether this concentration of D-glucose may affect the glucose carrier during the period of equilibrium binding of the ligands to the tumor cells. The tumor cells were incubated with PBS supplemented with 500 mM D-glucose at room temperature in a Thermolyne speci-mixer. Aliquots of the cell suspension were taken out at 0, 20 and 55 minutes after incubation. The tumor cells were harvested by centrifugation and washed twice with PBS. The washed tumor cells were then tested for glucose carrier. Table 1 shows that the number of glucose carrier (Bo) and the dissociation constant (K_d) of the tumor cells remained unchanged throughout the incubation with 500 mM D-glucose at different time intervals.

Cytochalasin B binding in the presence of other sugars and agents known to inhibit D-glucose transport was also examined by using 7 day-old tumor cells. Fig. 8A shows the binding curve of cytochalasin B in the presence and absence of 500 mM 2-deoxy-D-glucose which are comparable to that when D-glucose was added. The difference of cytochalasin B bound between the presence and absence of 500 mM 2-deoxy-D-glucose from Fig. 8A was similarly analysed by Scatchard plot. A best-fit linear plot was also obtained by linear regression (Fig. 8B). This plot represents 2-deoxy-D-glucose-sensitive cytochalasin B binding site on Ehrlich ascites tumor cells. It was estimated that the Bo was

about 218 pmol/10^7 cell and K_d value was 2.31 x 10^{-7} M. Fig. 9A and 9B show the cytochalasin B binding in the presence and absence of 500 mM 3-O-methyl-D-glucose as well as Scatchard plot of 3-O-methyl-D-glucose-sensitive cytochalasin B binding site. The values of Bo and K_d estimated from Scatchard plot are 205 pmol/10^7 cell and 2.24 x 10^{-7} M respectively. Similarly, the results of mannose, galactose, maltose are shown in Fig. 10A and 10B; 11A and 11B; and 12A and 12B respectively. The values of Bo and K_d for mannose are 144 pmol/10^7 cell and 1.53 x 10^{-7} M respectively. The values of Bo and K_d for galactose are 149 pmol/10^7 cell and 1.99 x 10^{-7} M respectively, and those for maltose are 153 pmol/10^7 cell and 2.00 x 10^{-7} M respectively. The results of two D-glucose uptake inhibitors, phloretin (5 x 10^{-5} M) and diethylstilbestrol (1 x 10^{-4} M), are shown in Fig. 13A and 13B; and Fig. 14A and 14B respectively. The values of Bo and K_d for phloretin are 215 pmol/10^7cell and 2.30 x 10^{-7} M respectively and those for diethylstilbestrol are 139 pmol/10^7 cell and 4.00 x 10^{-7} M respectively. However, when L-glucose was added, the binding curves of cytochalasin B remained unchanged disregarding the presence and absence of 500 mM L-glucose (Fig. 15). It is evident from Fig. 15 that L-glucose cannot displace cytochalasin B from its binding sites and therefore no Scatchard plot can be obtained. Similar binding curves of cytochalasin B with or without the addition of D-glucosamine, D-fructose, L-fucose, D-arabinose, sucrose and lactose are shown in Fig. 16, 17, 18, 19, 20 and 21 respectively. No displacement of cytochalasin B by these sugars could be observed. The specificity

TABLE 1. APPARENT DISSOCIATION CONSTANT (K_d) AND NUMBER OF GLUCOSE CARRIER (Bo) IN EHRLICH ASCITES TUMOR CELLS AFTER INCUBATION WITH 500 mM D-GLUCOSE[a]

Time after incubation with 500 mM D-glucose (minutes)	Number of glucose carrier (Bo) (pmol/10^7 cell)	Apparent dissociation constant of glucose carrier (K_d) (10^{-7} M)
0	215	2.66
20	191	2.17
55	193	2.52

a. Cells from 7 day-old tumors were used. The difference in binding in the presence and in the absence of 500 mM D-glucose was analysed by Scatchard plot. Numbers represent the mean values of triplicate sample determination.

Fig. 8 Capacity of Ehrlich ascites tumor cells to bind cytochalasin B in the presence of 2-deoxy-D-glucose.

A. Binding curves of cytochalasin B by 7 day-old Ehrlich ascites tumor cells as a function of 2-deoxy-D-glucose concentration. Percentage of cytochalasin B bound in the absence (● - ●) and in the presence (○ - ○) of 500 mM 2-deoxy-D-glucose are shown. The difference represents the 2-deoxy-D-glucose-sensitive portion.

B. Scatchard analysis of the 2-deoxy-D-glucose-sensitive binding of cytochalasin B to 7 day-old Ehrlich ascites tumor cells determined from Fig. 8A. Control data from Fig. 8A were used to calculate the bound and free ligand. The line was the best fit according to linear least squares analysis. Total binding site (Bo) and dissociation constant (Kd) for 2-deoxy-D-glucose-sensitive cytochalasin B binding site on Ehrlich tumor cells were determined as 218 pmol/10^7 cell and 2.3×10^{-7} M respectively.

FIGURE 8A

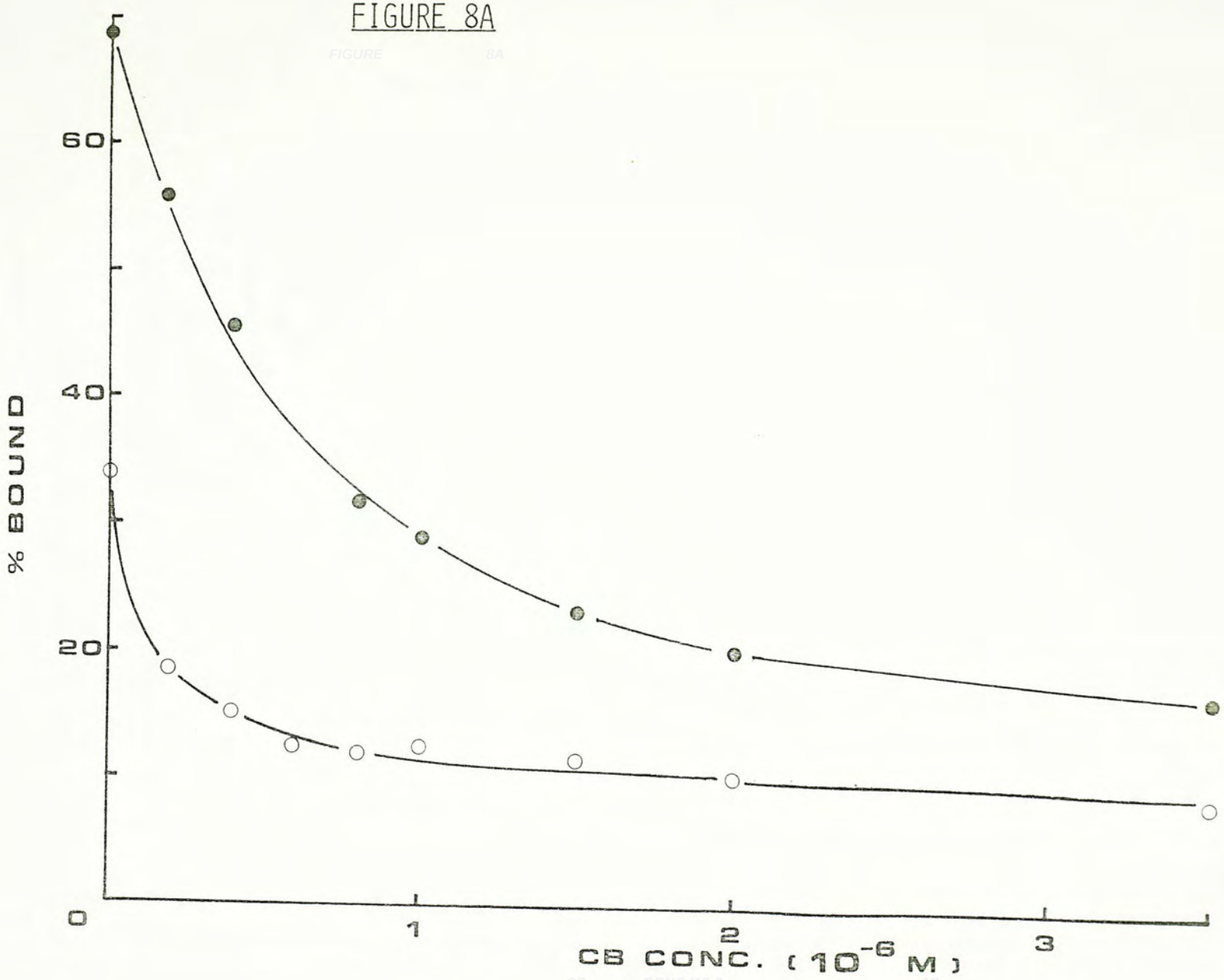


FIGURE 8B

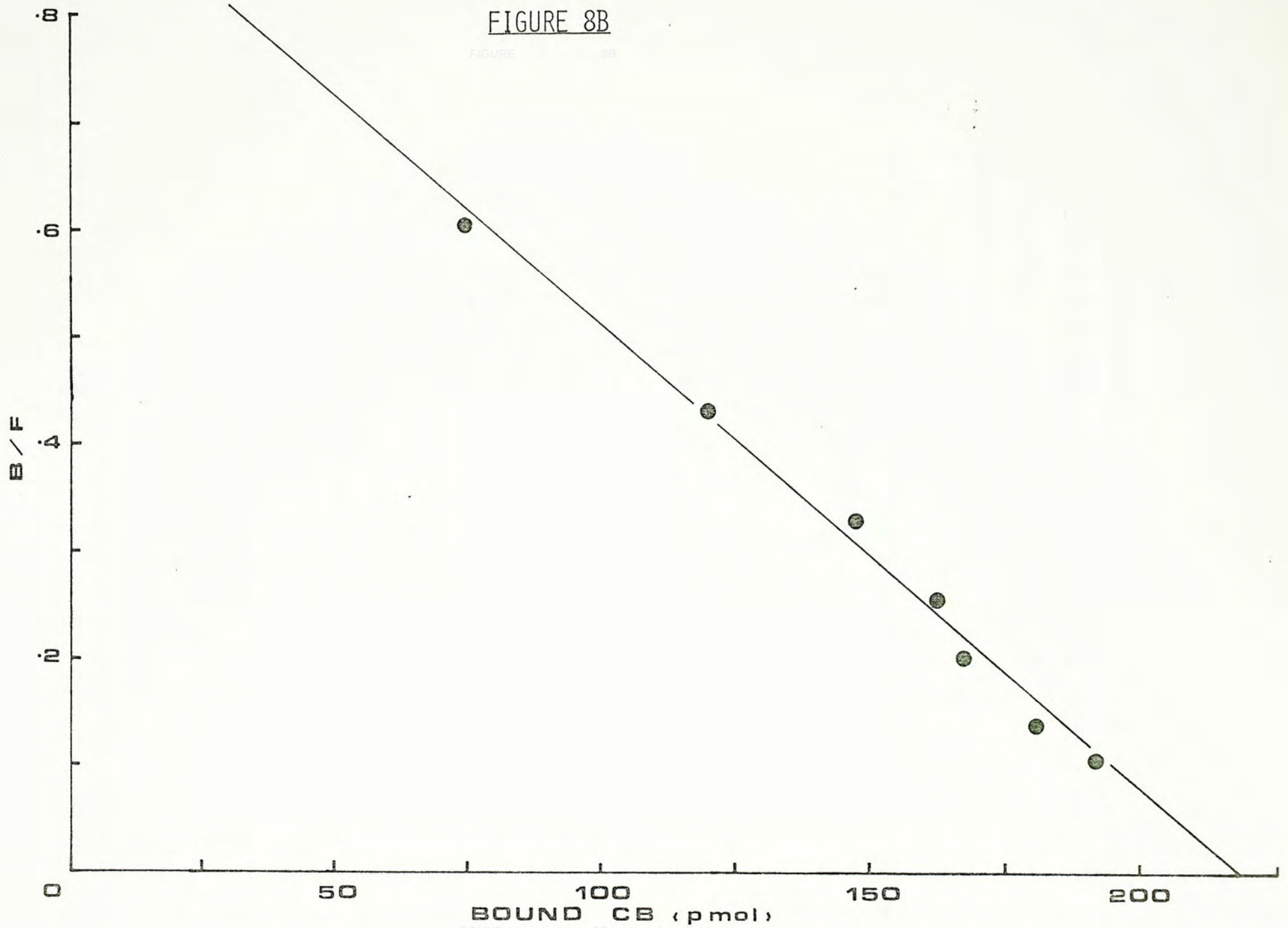


Fig. 9 - Fig. 14

Capacity of 7 day-old Ehrlich ascites tumor cells to bind cytochalasin B in the presence of various sugars and two glucose uptake inhibitors, phloretin (5×10^{-5} M) and diethylstilbestrol (1×10^{-4} M):

A. Binding curve of cytochalasin B by 7 day-old Ehrlich ascites tumor cells as a function of ligand concnetration.

B. Scatchard analysis of the sugars/glucose uptake inhibitors-sensitive binding of cytochalasin B to 7 day-old Ehrlich ascites tumor cells determined from A part of the figure. Control data from A part of the figure were used to calculate the bound and free ligand. The lines were the best fit according to linear least squares analysis. Total binding site (Bo) and dissociation constnat (Kd) for various sugars/glucose uptake inhibitors-sensitive cytochalasin B binding site on Ehrlich asictes tumor cells can thus be determined.

Fig. 9

A. Binding curves of cytochalasin B in the absence of (●-●) and in the presence (○-○) of 500 mM 3-O-methyl-D-glucose.

B. Scatchard analysis of 3-O-methyl-D-glucose-sensitive binding of cytochalasin B to Ehrlich ascites tumor cells determined from Fig. 9A.

Fig. 10

A. Binding curves of cytochalasin B in the absence of (●-●) and in the presence (✱-✱) of 500 mM D-mannose.

B. Scatchard analysis of D-mannose-sensitive cytochalasin B binding to Ehrlich ascites tumor cells determined from Fig. 10A.

Fig. 11

A. Binding curves of cytochalasin B in the absence of (●-●) and in the presence (○-○) of 500 mM D-galactose.

B. Scatchard analysis of D-galactose-sensitive cytochalasin B binding to Ehrlich ascites tumor cells determined from Fig. 11A.

FIGURE 9A

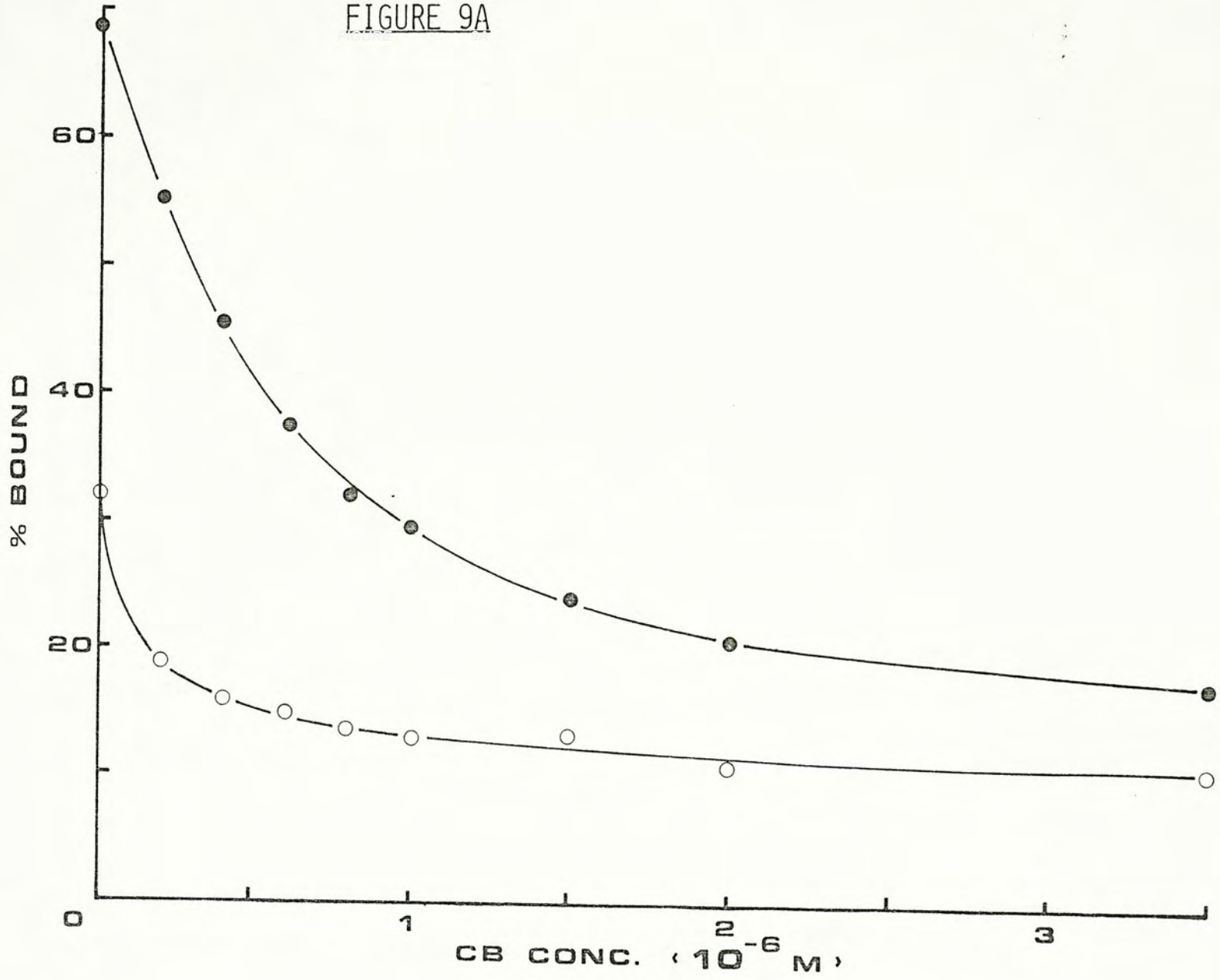


FIGURE 9B

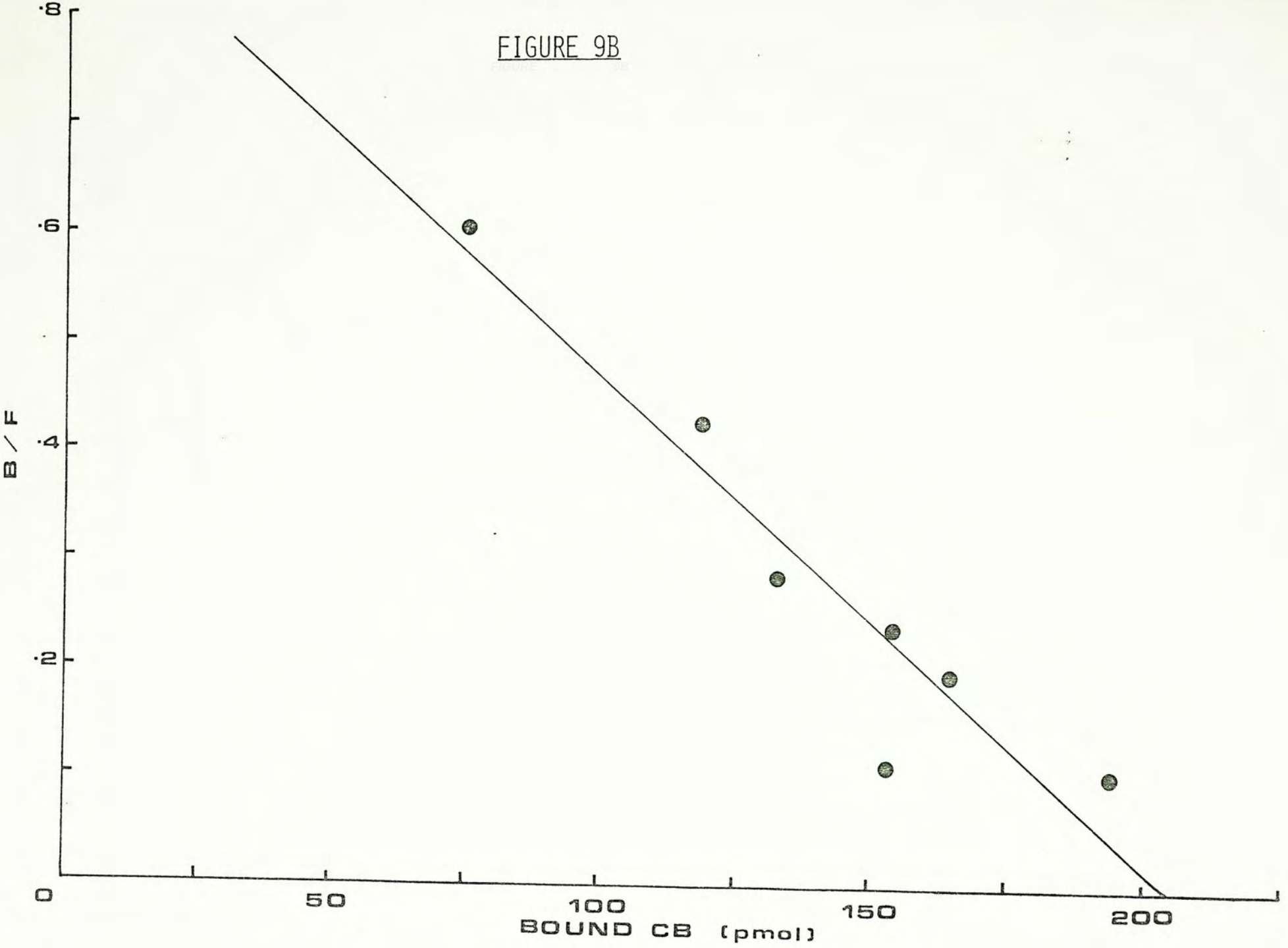


FIGURE 10A

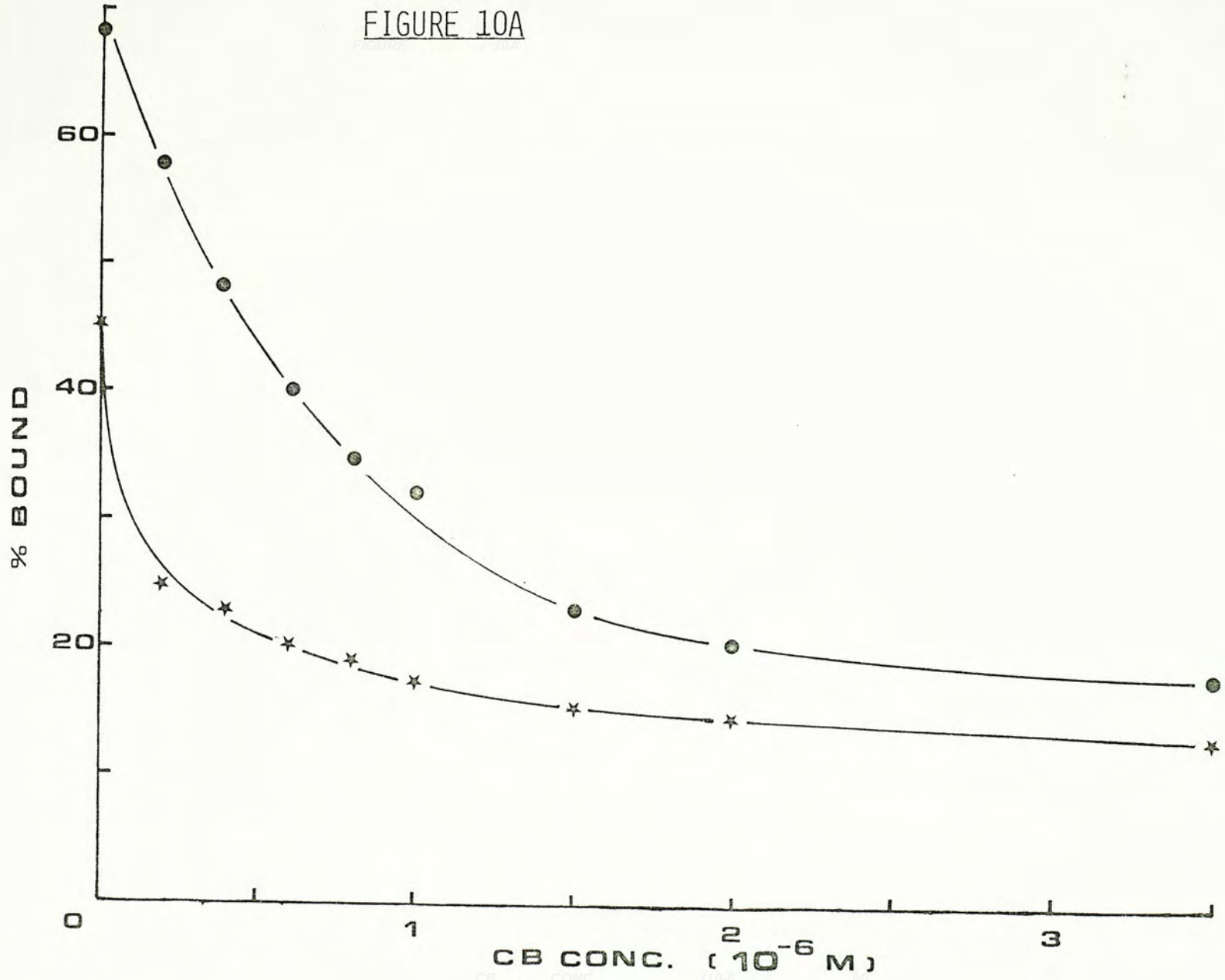


FIGURE 10B

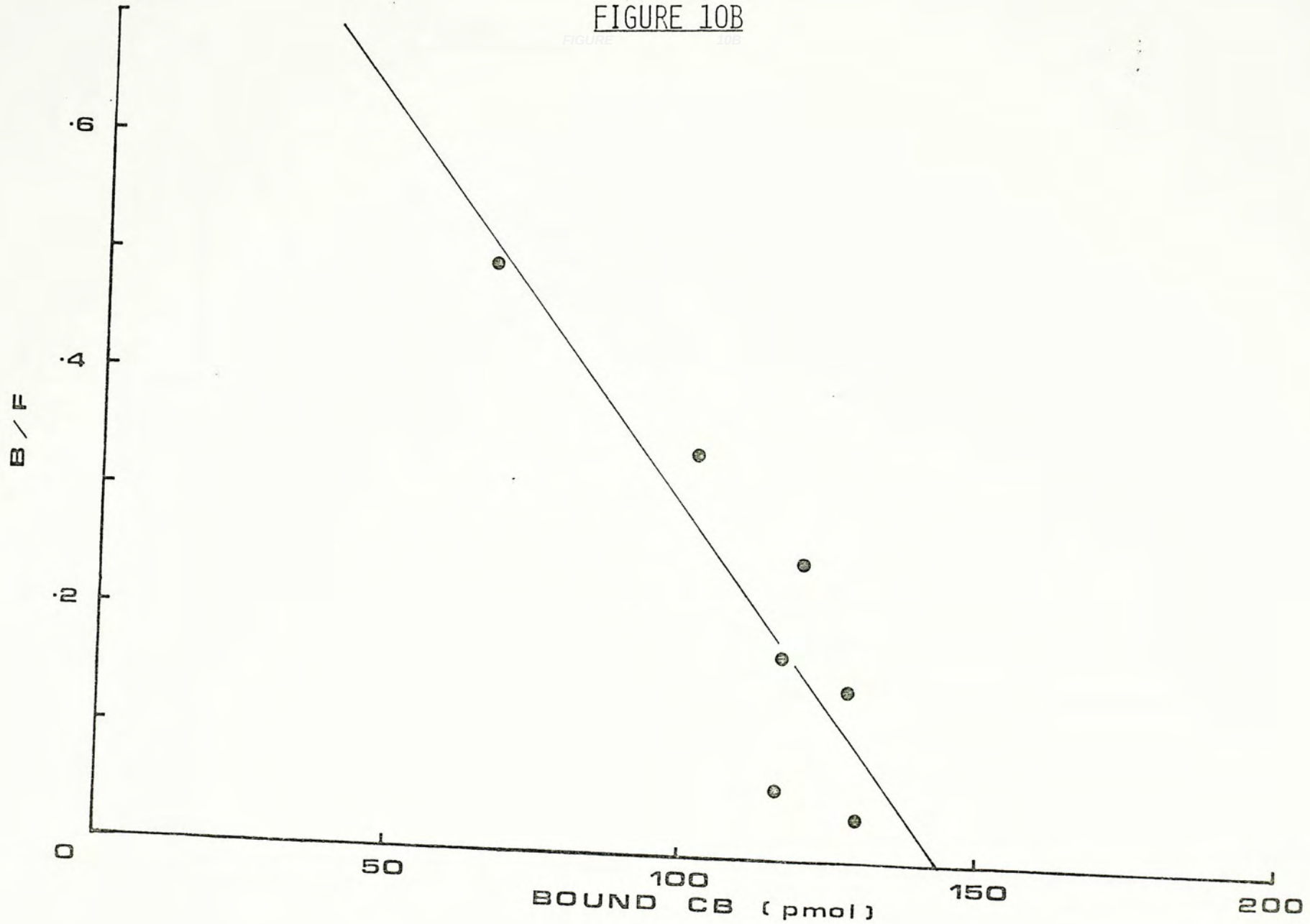


FIGURE 11A

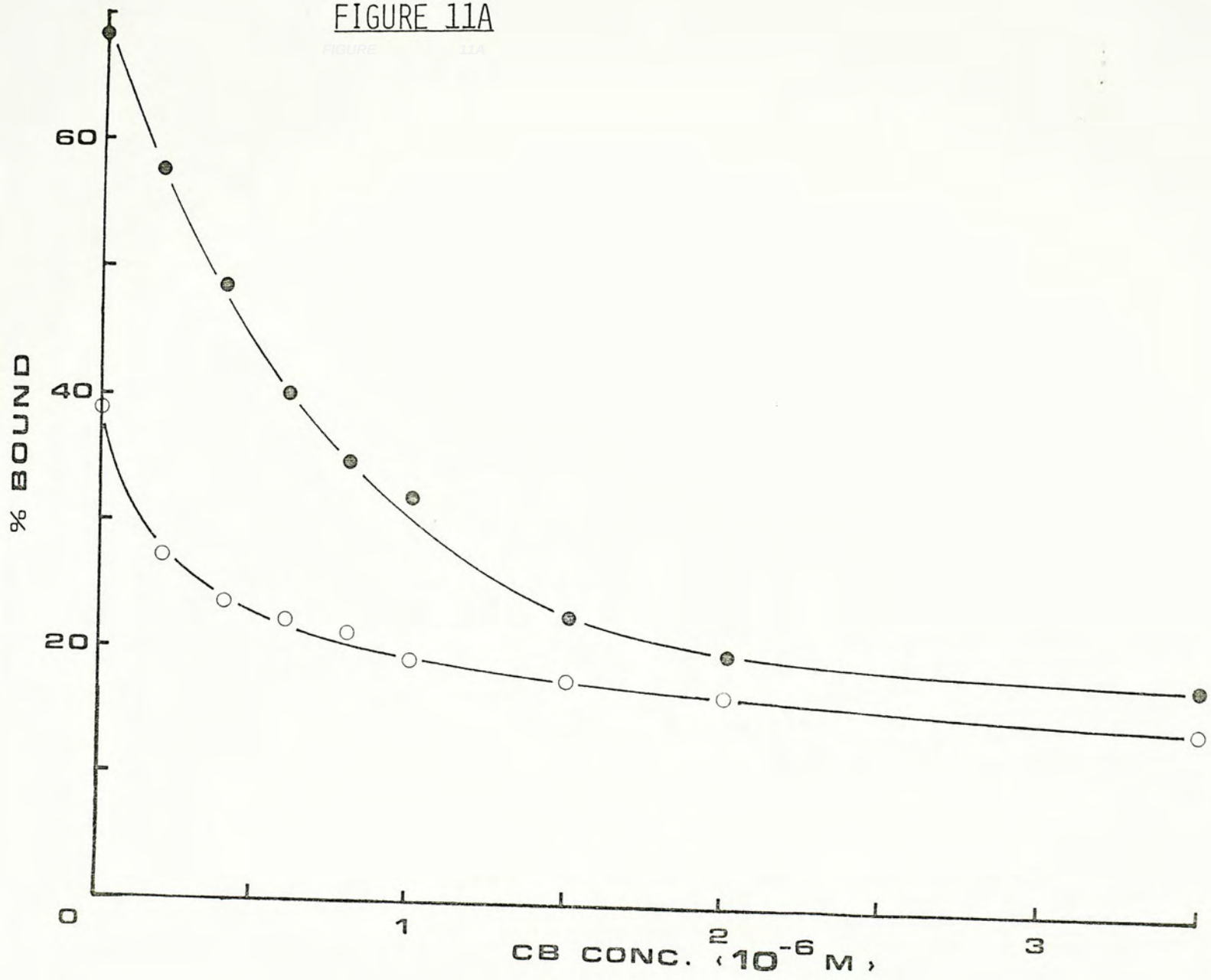


FIGURE 11B

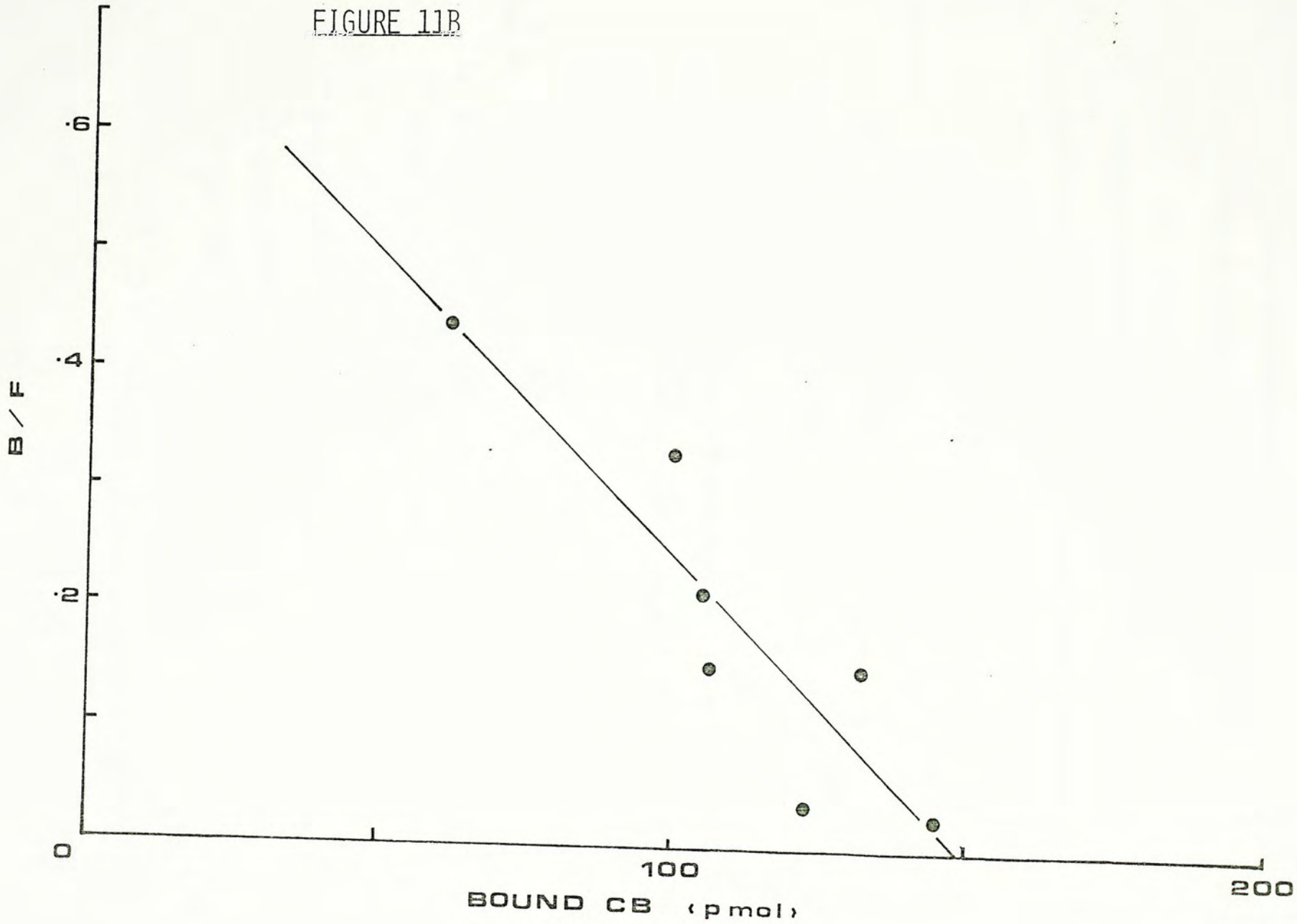


Fig. 12

A. Binding curves of cytochalasin B in the absence (● - ●) and in the presence (○ - ○) of 500 mM maltose.

B. Scatchard analysis of maltose-sensitive binding of cytochalasin B to Ehrlich ascites tumor cells determined from Fig. 12A.

Fig. 13

A. Binding curves of cytochalasin B in the absence (● - ●) and in the presence (○ - ○) of 5×10^{-5} M phloretin.

B. Scatchard analysis of phloretin-sensitive binding of cytochalasin B to Ehrlich ascites tumor cells determined from Fig. 13A.

Fig. 14

A. Binding curves of cytochalasin B in the absence (● - ●) and in the presence (○ - ○) of 1×10^{-4} M diethylstilbestrol.

B. Scatchard analysis of diethylstilbestrol-sensitive binding of cytochalasin B to Ehrlich ascites tumor cells determined for Fig. 14A.

FIGURE 12A

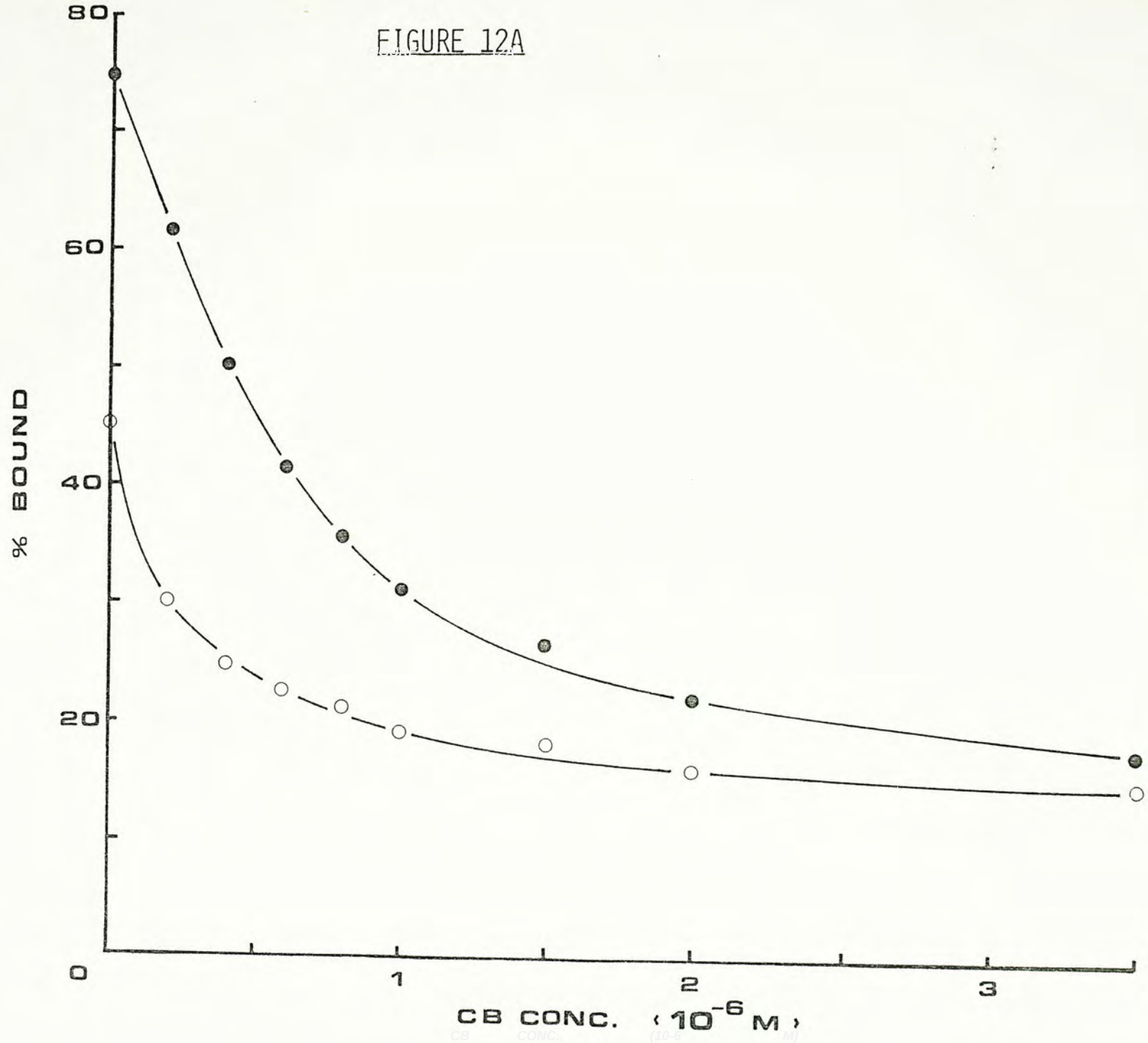


FIGURE 12B

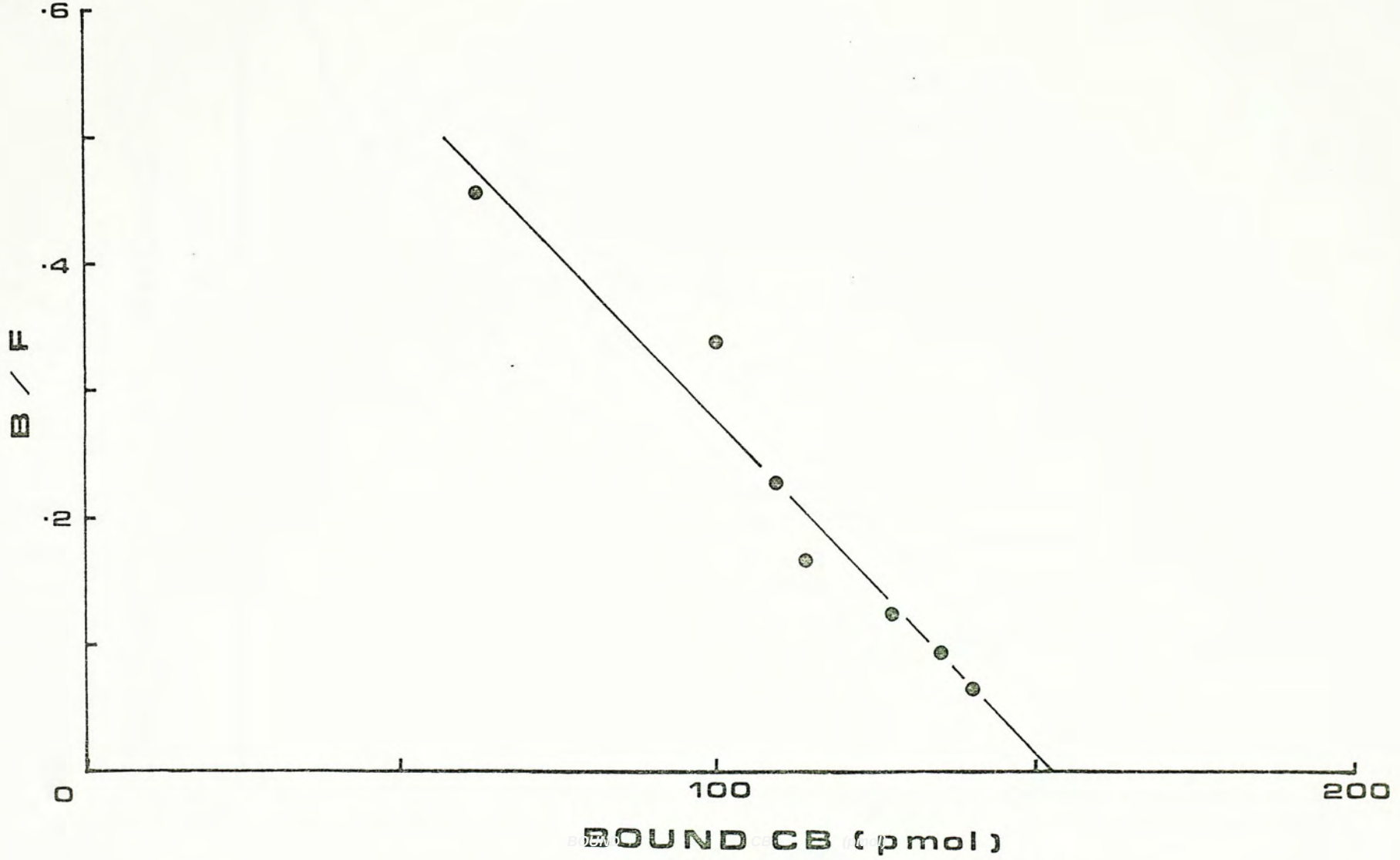


FIGURE 13A

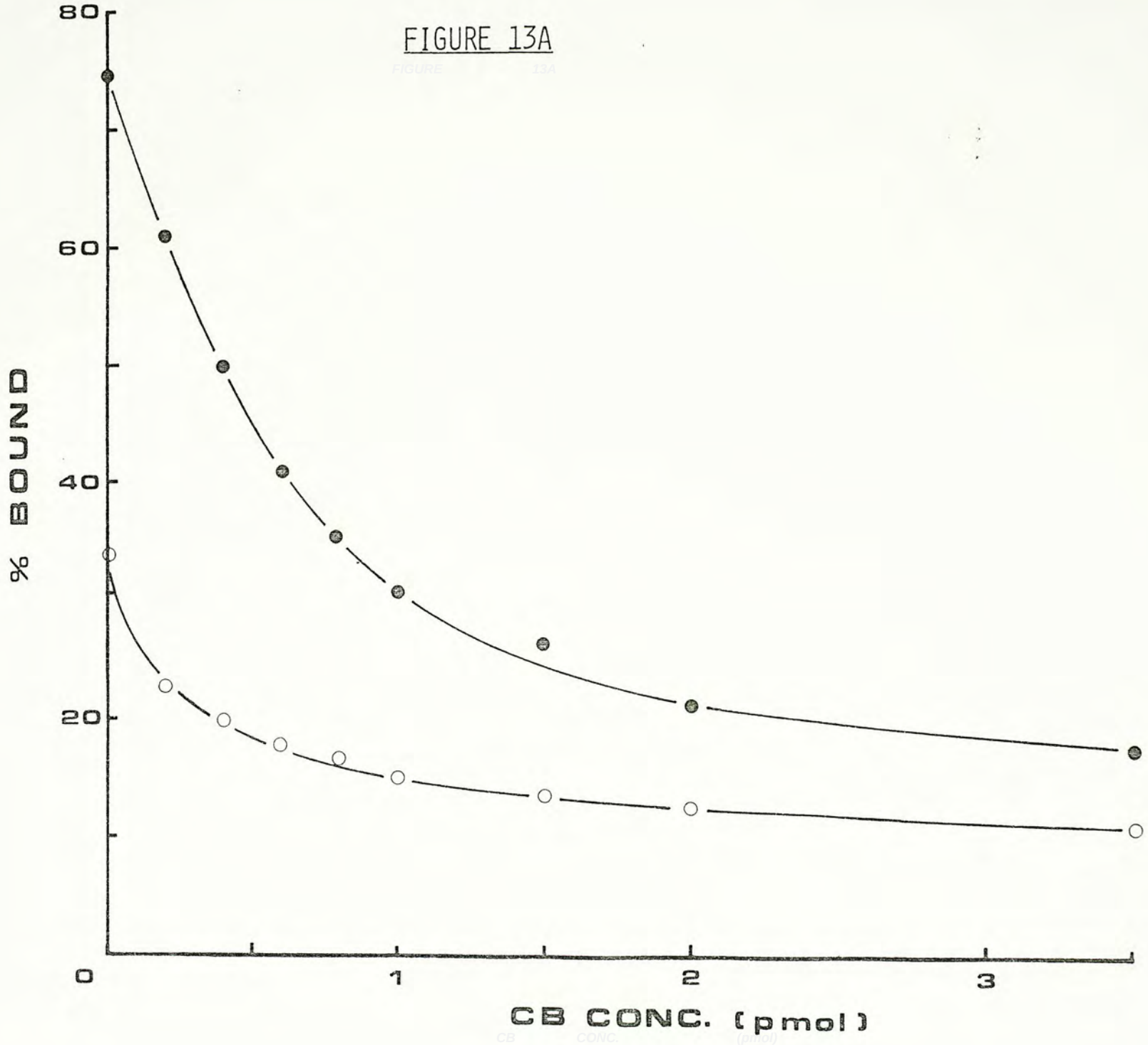


FIGURE 13B

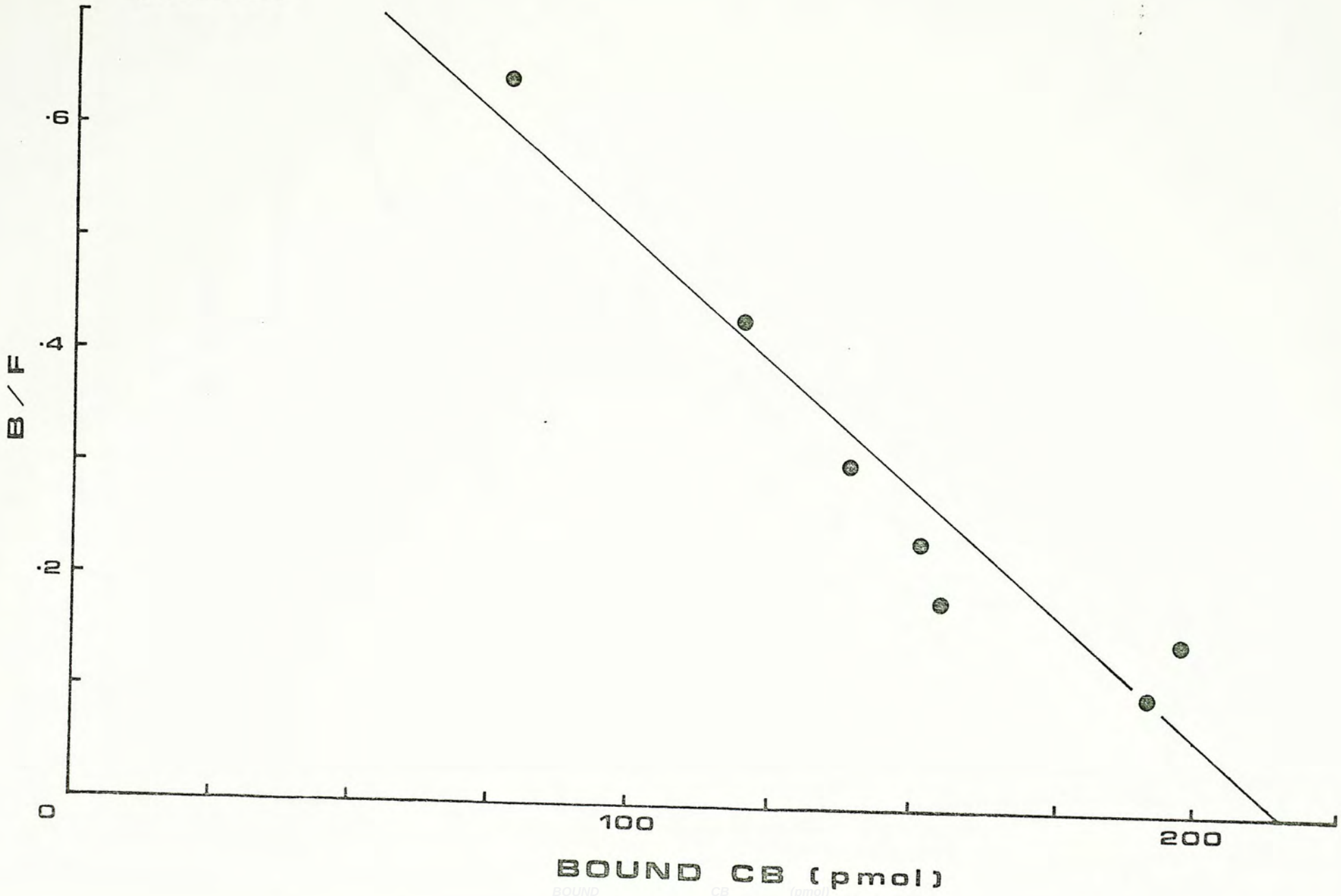


FIGURE 14A

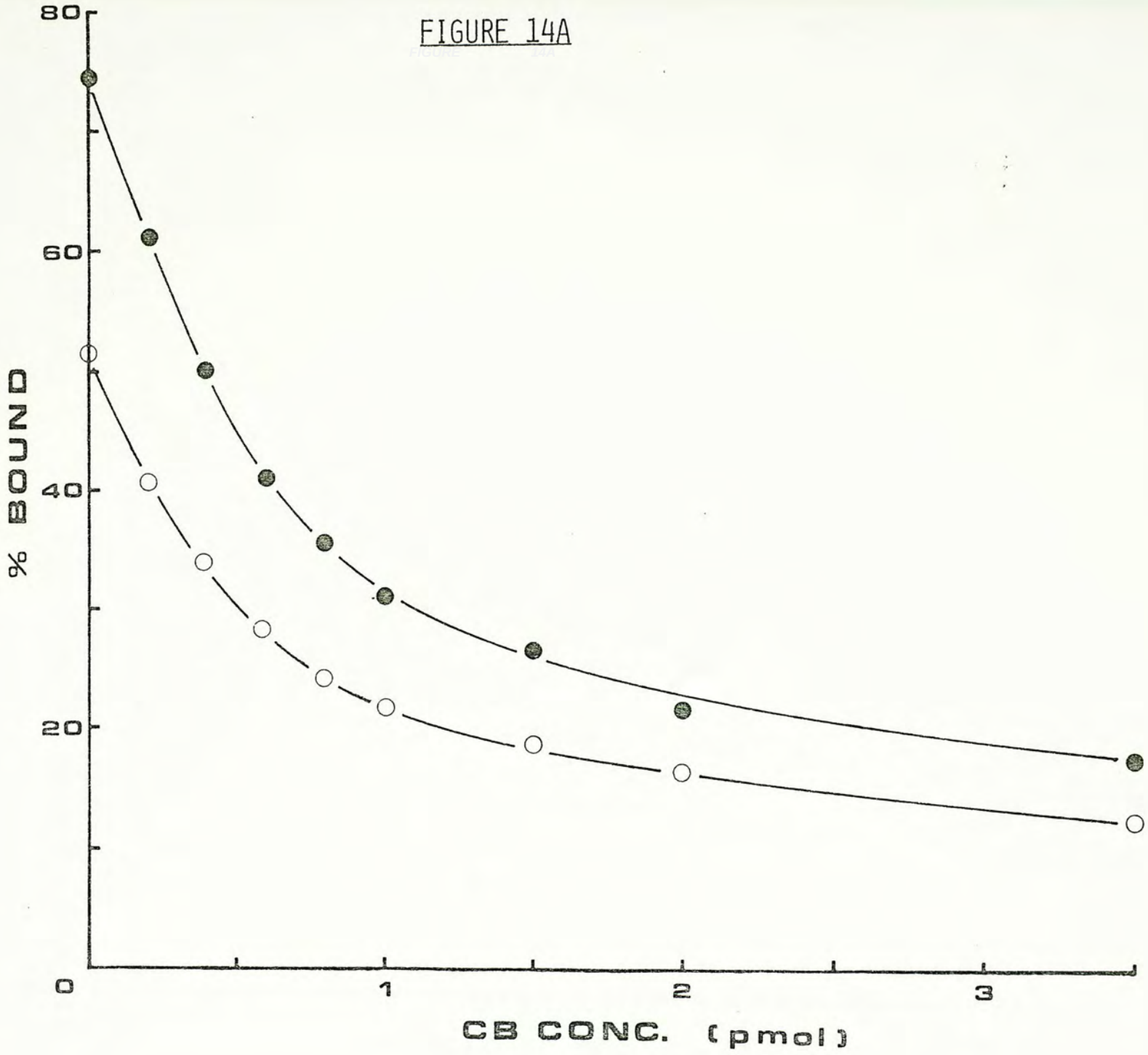


FIGURE 14B

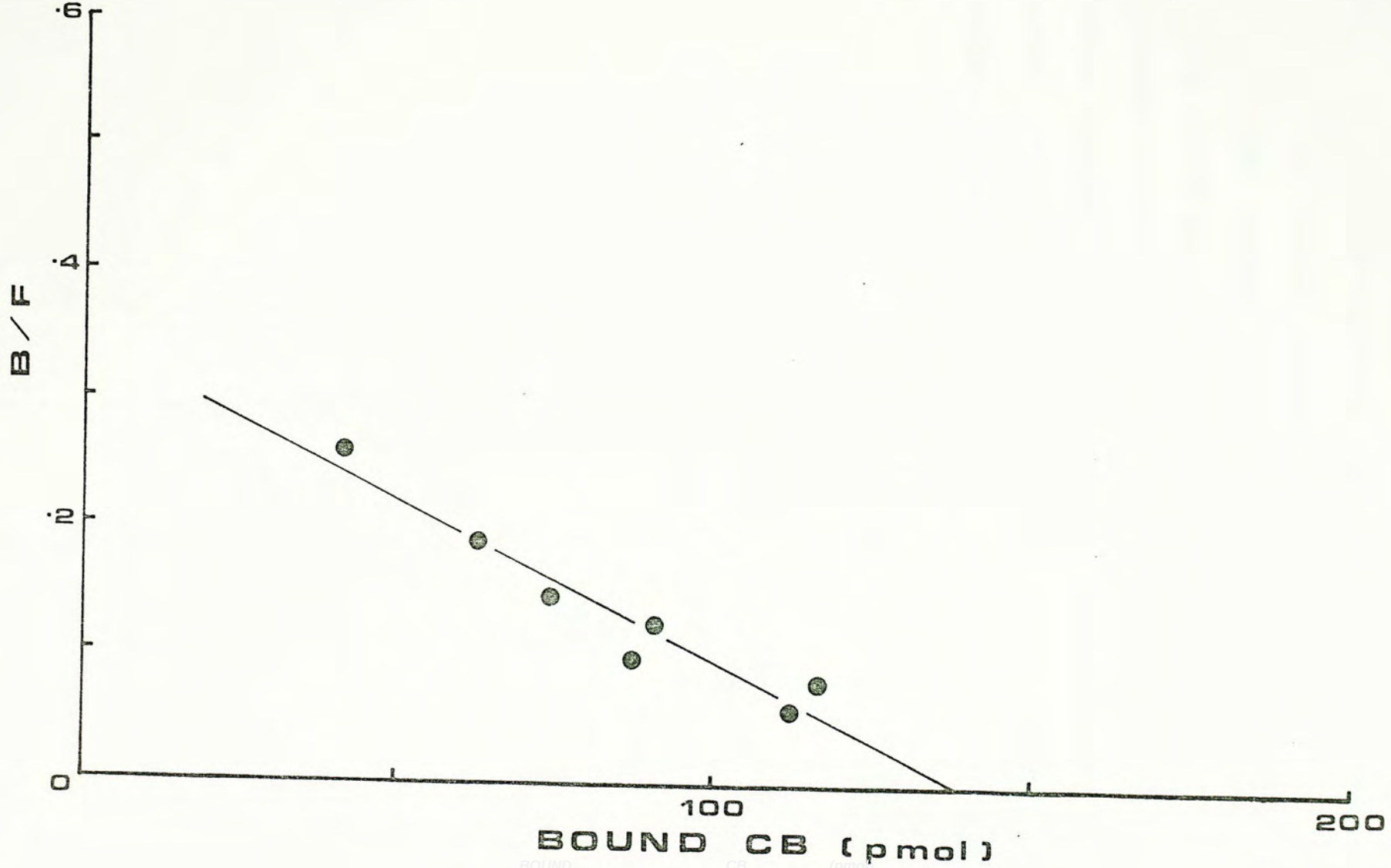


Fig. 15 Capacity of Ehrlich ascites tumor cells to bind cytochalasin B in the presence of L-glucose. The binding curves of cytochalasin B by 7 day-old tumor cells as a function of ligand concentration. Percentage of cytochalsin B bound in the absence (★-★) and in the presence (☆-☆) of 500 mM L-glucose are shown. There is essentially no difference between these two curves. Therefore no L-glucose-sensitive portion of cytochalasin B binding and thus no Scatchard analysis can be constructed.

FIGURE 15

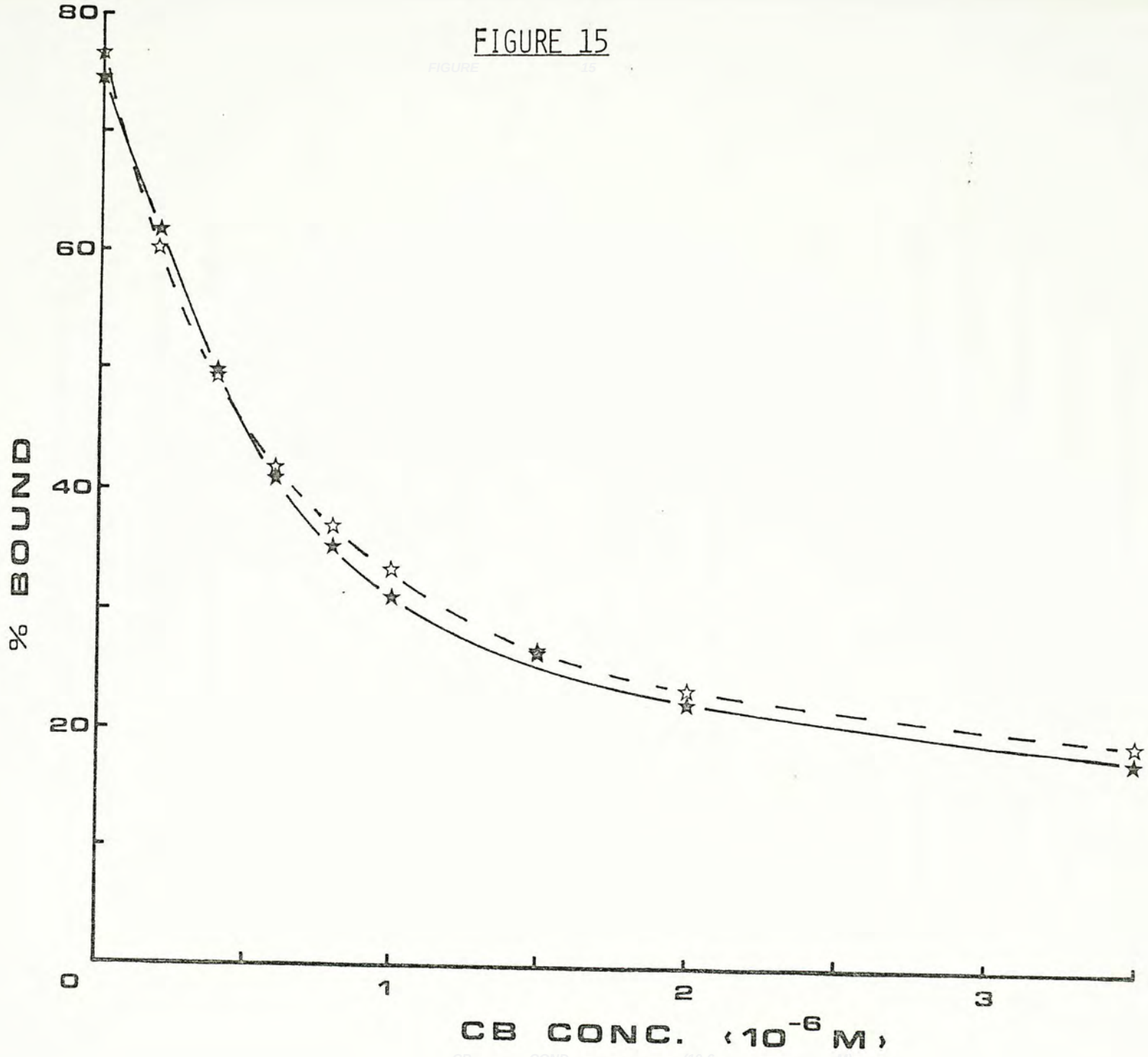


Fig. 16 - Fig. 21

Capacity of Ehrlich ascites tumor cells to bind cytochalasin B in various sugars. The binding curve of cytochalasin B by 7 day-old Ehrlich ascites tumor cells as a function of ligand concentration. Since these sugars cannot displace cytochalasin B from its binding sites, no Scatchard analysis of the sugars-sensitive cytochalasin B binding site can be constructed.

Fig. 16 Binding curves of cytochalasin B in the absence (★-★) and in the presence (☆-☆) of 500 mM D-glucosamine. This is essentially no difference between these two binding curves.

Fig. 17 Binding curves of cytochalasin B in the absence (★-★) and in the presence (☆-☆) of 500 mM L-fucose. There is essentially no difference between these two binding curves.

Fig. 18 Binding curves of cytochalasin B in the absence (★-★) and in the presence (☆-☆) of 500 mM D-fructose. The difference between these two binding curves is small and causes difficulties in constructing Scatchard analysis. Therefore fructose is regarded as a sugar which cannot displace cytochalasin B from its binding sites.

FIGURE 16

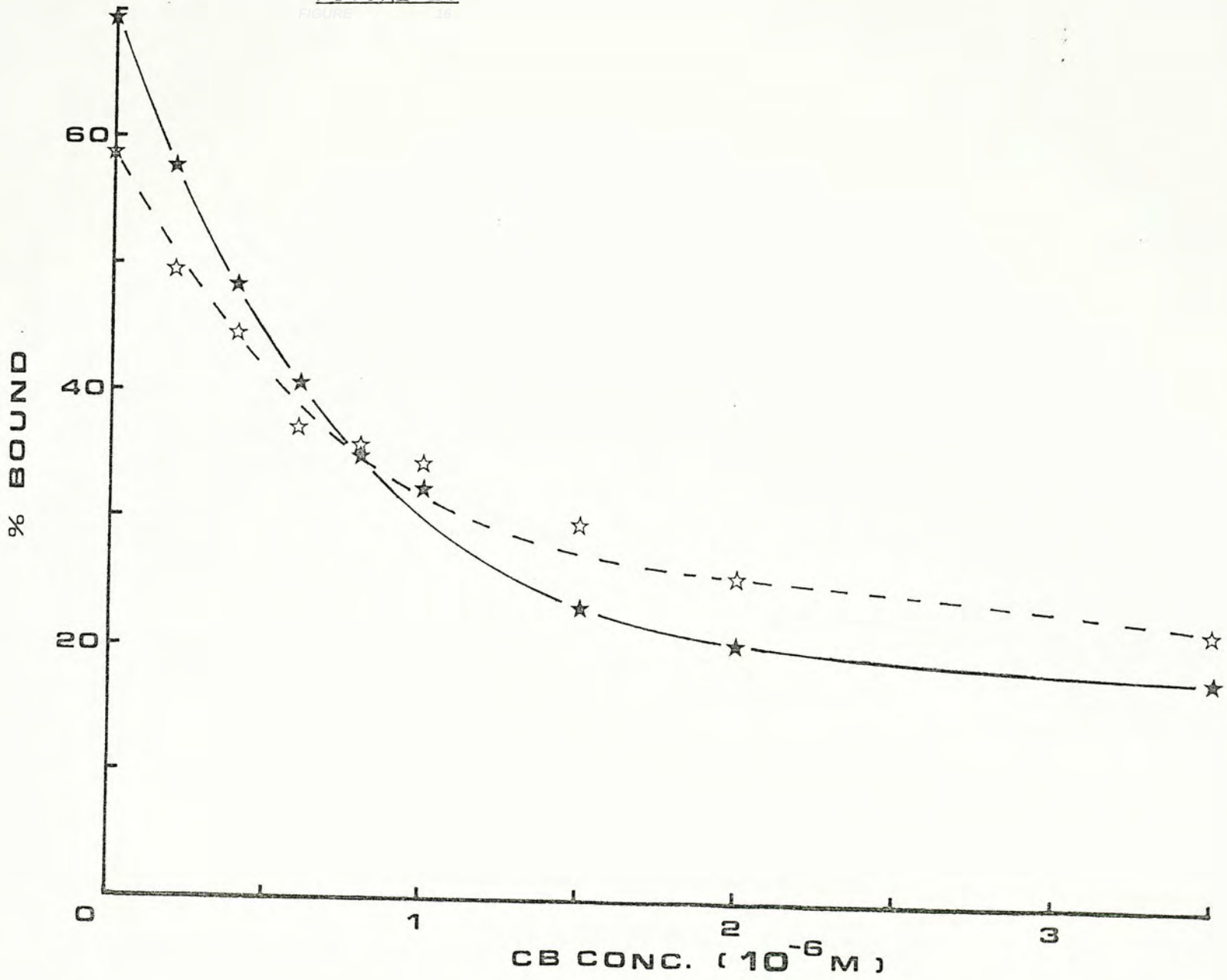


FIGURE 17

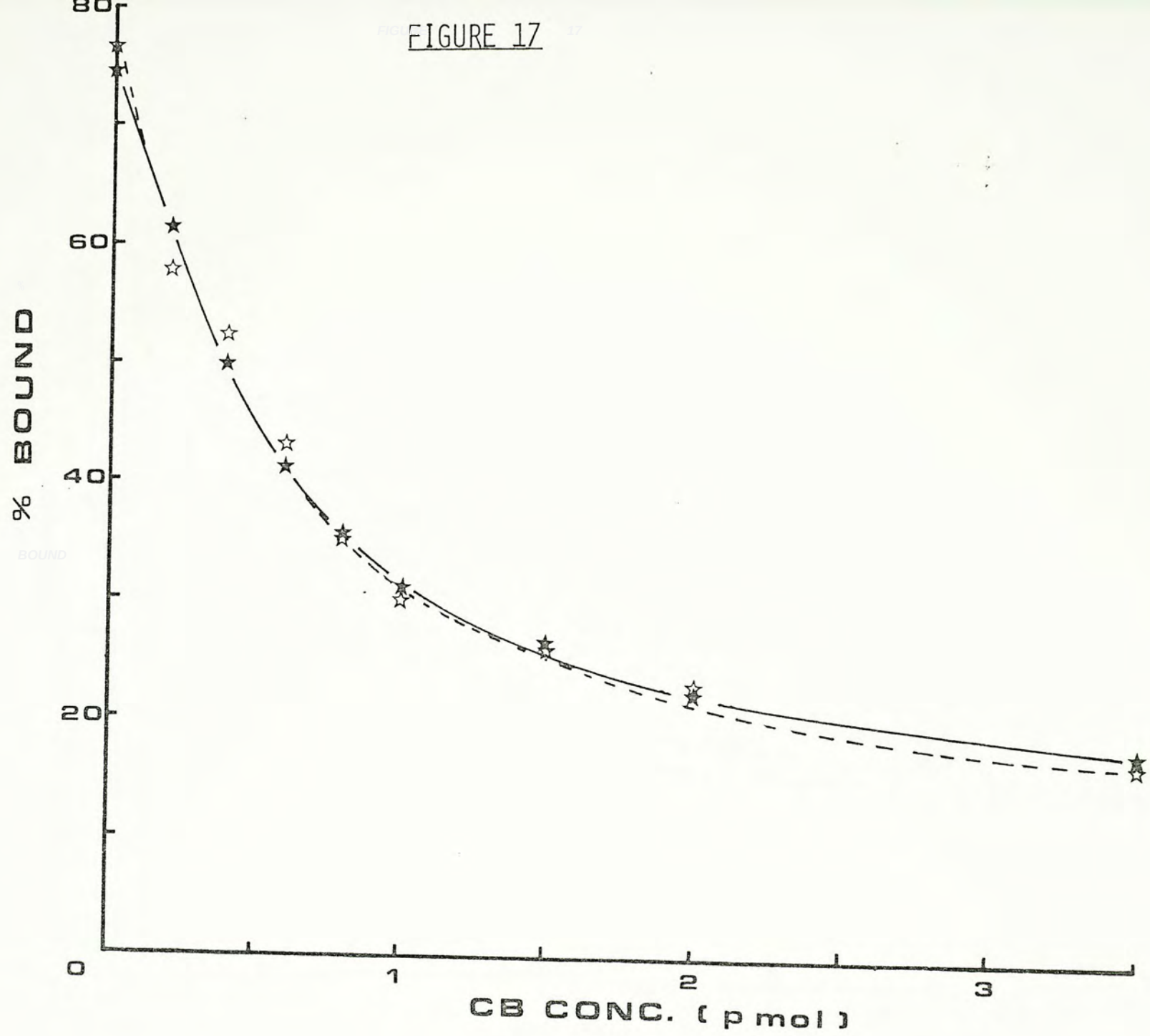


FIGURE 18

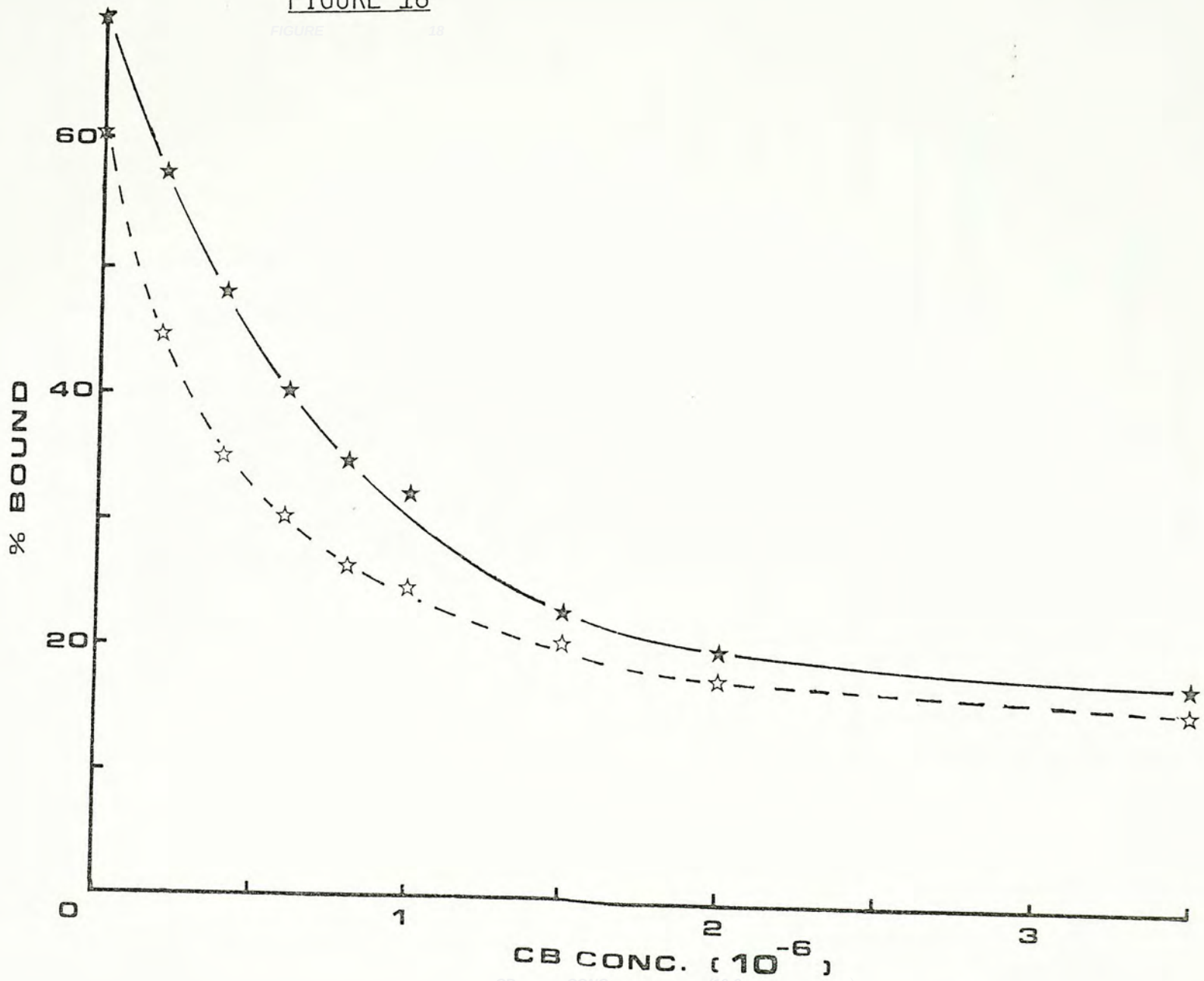


Fig. 19 Binding curves of the cytochalasin B in the absence (★-★) and in the presence (☆-☆) of 500 mM D-arabinose. There is essentially no difference between these two binding curves.

Fig. 20 Binding curves of cytochalasin B in the absence (★-★) and in the presence (☆-☆) of 500mM sucrose. There is essentially no difference between these two binding curves.

Fig. 21 Binding curves of cytochalasin B in the absence (★-★) and in the presence (☆-☆) of 500 mM lactose. There is essentially no difference between these two binding curves.

FIGURE 19

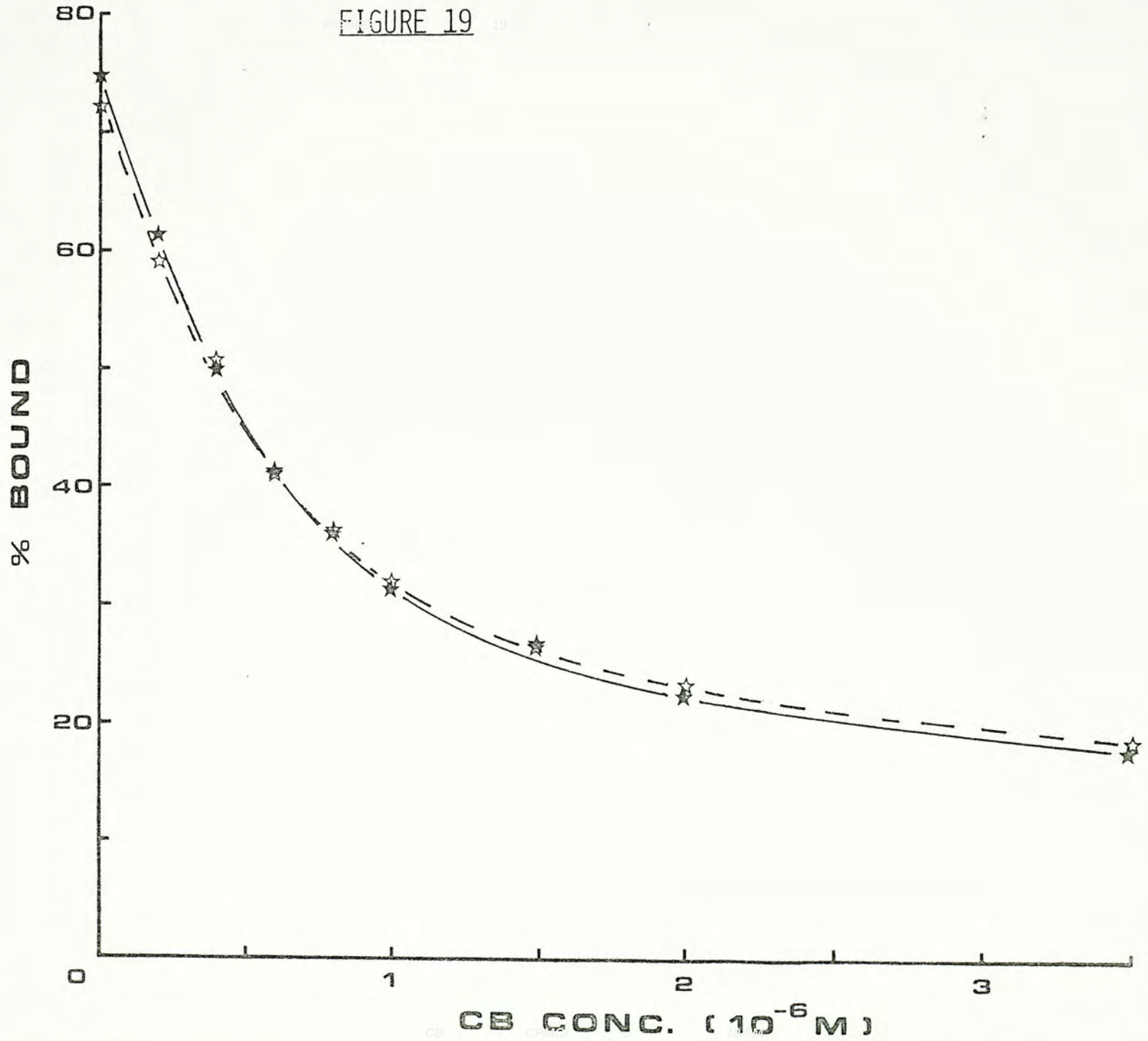


FIGURE 20

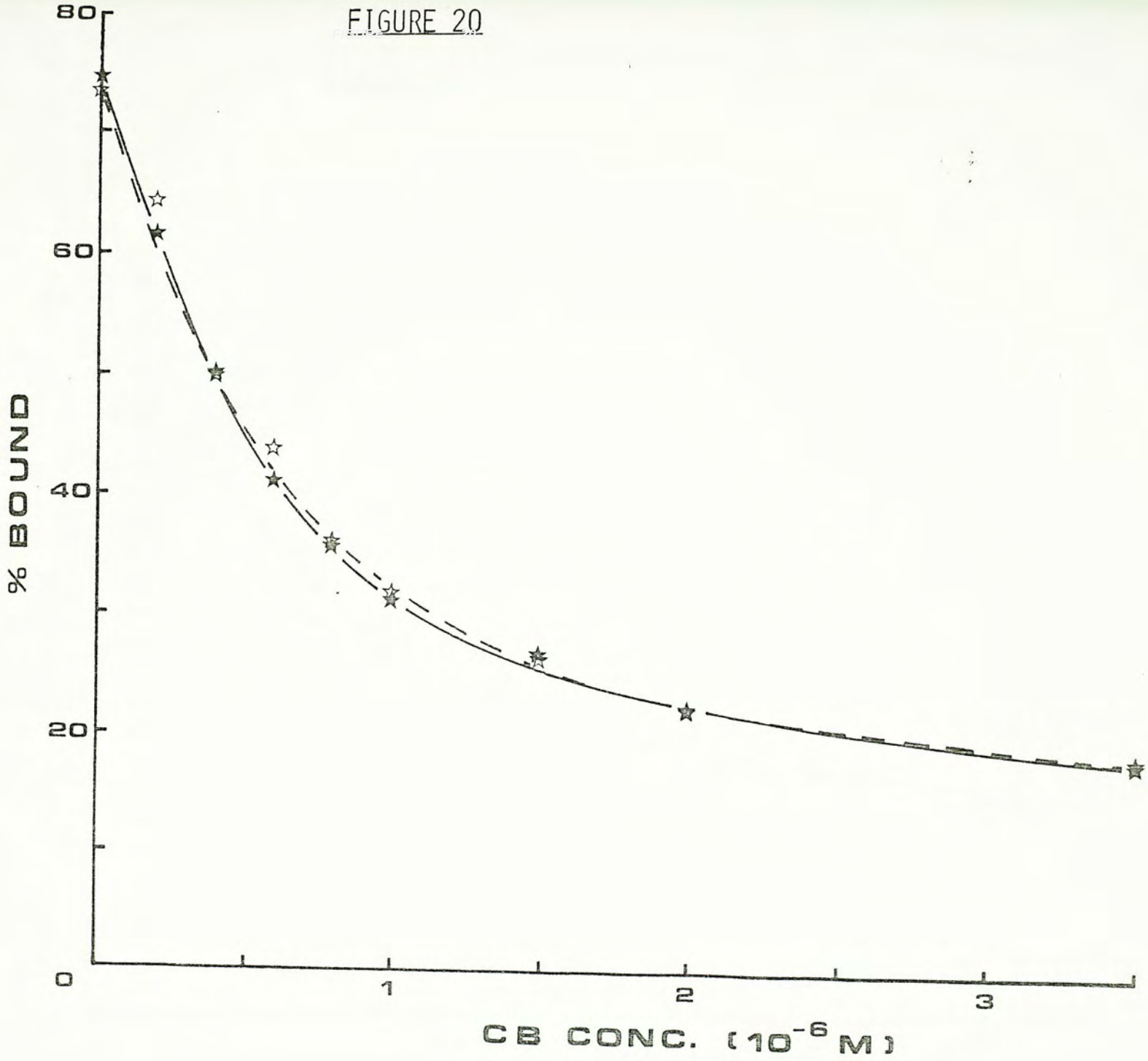


FIGURE 21

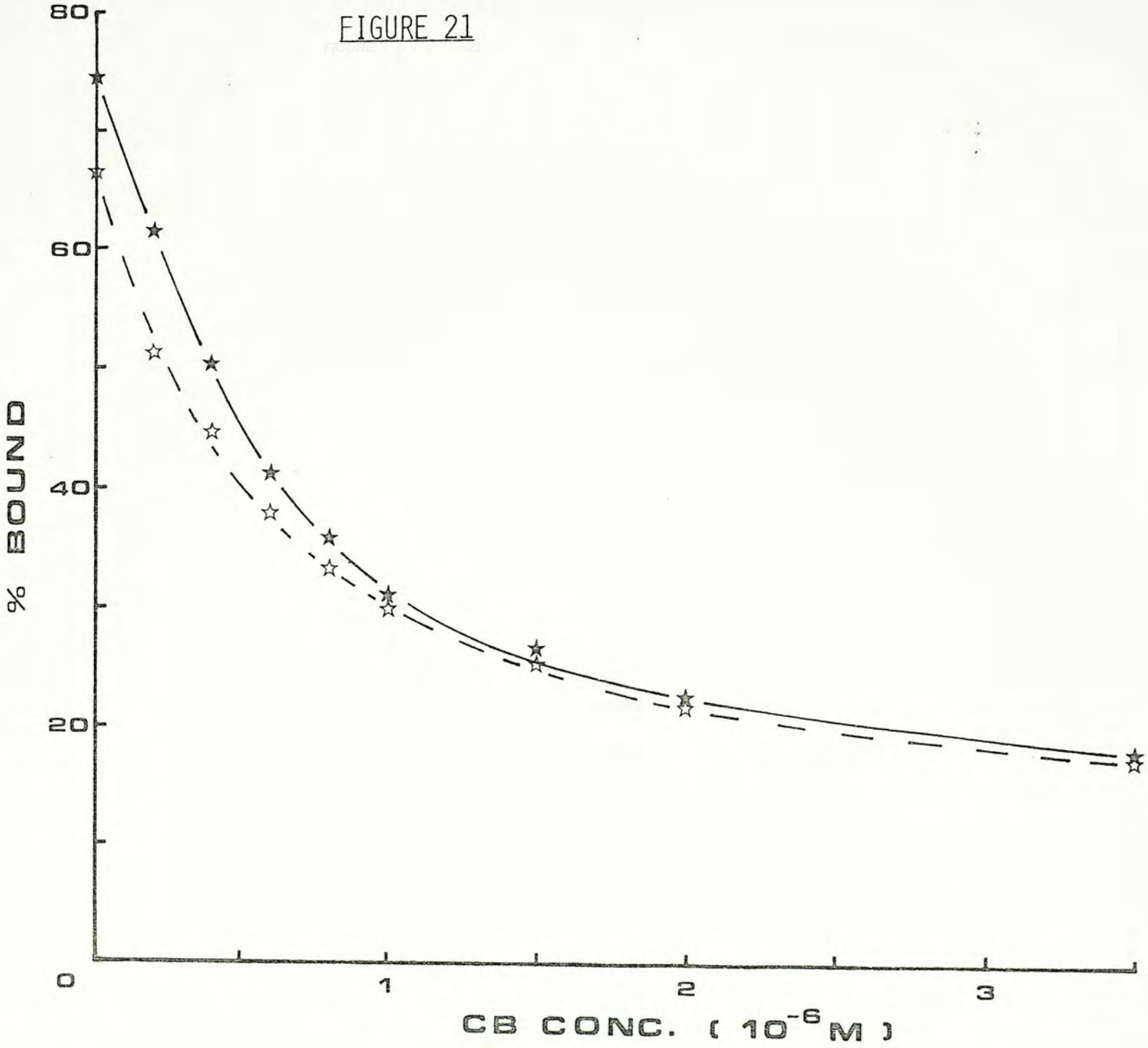


TABLE 2. APPARENT DISSOCIATION CONSTANT (K_d) AND NUMBER OF BINDING SITE (Bo) FOR CYTOCHALASIN B ON EHRLICH ASCITES TUMOR CELLS[a]

LIGANDS[b]	COMPETITIVITY[c]	Bo (pmol/10^7 cell)	K_d (10^{-7} M)
D-Glucose	+	215	2.6
2-Deoxy-D-glucose	+	218	2.3
3-O-Methyl-D-glucose	+	205	2.2
D-Galactose	+	149	2.0
D-Mannose	+	144	1.5
Maltose	+	153	2.0
Phloretin	+	215	2.3
Diethylstilbestrol	+	139	4.0
D-Glucosamine	-	/	/
D-Fructose	-	/	/
L-Fucose	-	/	/
D-Arabinose	-	/	/
Sucrose	-	/	/
Lactose	-	/	/
L-Glucose	-	/	/

a. D-glucose- and ligand-reversible binding of cytochalasin B was measured as described in the text. Cells from 7 day-old tumors were used. The difference in the binding in the presence and in the absence of competing ligands was analysed by Scatchard plot. Numbers are presented as the mean values of triplicate sample determination.

b. The final sugar concentrations were 500 mM, those of phloretin and diethylstilbestrol were respectively 5×10^{-5} M and 10^{-4} M.

c. Ligands which cannot displace cytochalasin B from glucose carriers are regarded as no competitivity with cytochalasin B in the binding to glucose carriers.

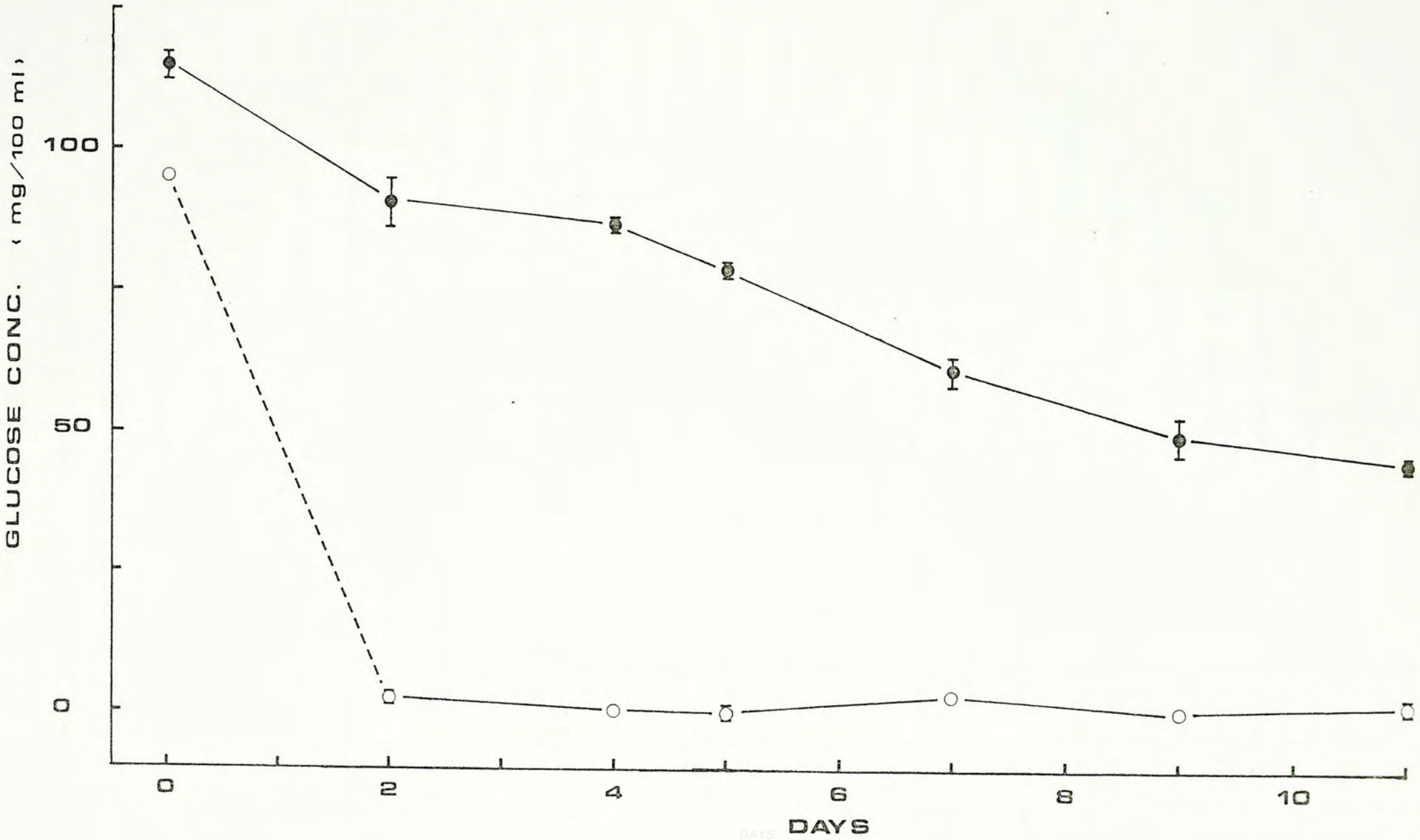
of the glucose carrier are summarized in Table 2. The specificity of the glucose carrier is rather high, only two D-glucose derivatives, 2-deoxy-D-glucose and 3-O-methyl-D-glucose can substitute D-glucose and completedly displace cytochalasin B from the glucose carrier. Another two hexose, mannose and galactose, having very similar structures with D-glucose can only partially displace cytochalasin B from the glucose carrier. On the other hand, among the two D-glucose uptake inhibitors, phloretin can completedly while diethylstilbestrol can only partially displace cytochalasin B from the glucose carrier. However the dimer of glucose, maltose, can also partially displace cytochalasin B from glucose carrier. By contrast, other hexose like fructose; disaccharides like sucrose and lactose; pentose like arabinose; amino sugar like glucosamine cannot displace cytochalasin B from glucose carrier. Furthermore, this glucose carrier is stereospecific for D-glucose since L-glucose cannot displace cytochalasin B from this carrier molecule. L-fucose cannot displace cytochalasin B from glucose carrier too.

VI. Effect of serum glucose on the number of glucose carrier in Ehrlich ascites tumor cells *in vivo*

The changes in serum glucose and the glucose level of ascites fluid during tumor development are shown in Fig. 22. In examining the serum glucose of tumor bearing mice, it was found that hypoglycemia appeared during the development of tumor. This result agrees with the findings of other tumor

Fig. 22 Concentration of glucose in mouse serum and ascites fluid during tumor development. Mice were inoculated with 10^7 tumor cells on day 0. Glucose levels in serum (● - ●) and ascites fluid (○ - ○) were determined on indicated days post transplantation. Values are expressed as the mean ± S.E.M. for three separate experiments. Blood samples were obtained from mice after overnight fasting. Values of day 0 represent the relative glucose levels of normal mice. The glucose level in peritoneal fluid of normal mice was obtained from the result of Nakamura and Hosada (1968). The broken line is the estimation of the change in glucose level in peritoneal fluid from day 0 to day 2.

FIGURE 22

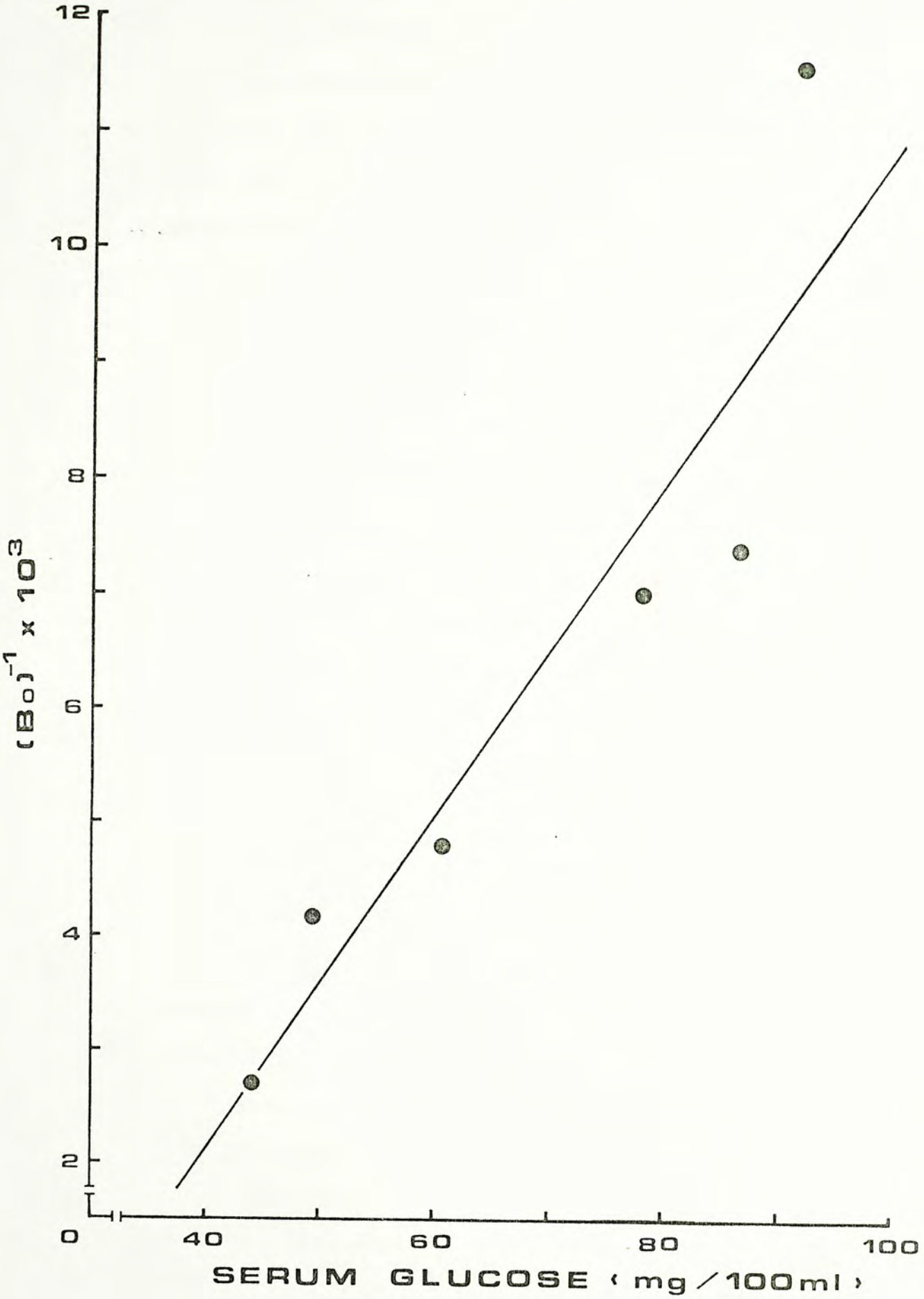


cell lines (Vaissman *et al* 1964; Goranson *et al* 1954; Goranson and Tilser 1955; Tagi-Zade and Shapot 1971). The serum glucose fell from 115 mg/100 ml on day 0 to 44 mg/100 ml on day 11 post transplantation. By contrast, there was essentially no measurable glucose in the ascites fluid from day 2 to day 11 after the tumor inoculation and this result is consistent with the finding of Nakamura and Hosoda (1968). It should be noted that during the development of tumors, serum glucose of the hosts decreased progressively but by contrast the number of glucose carrier in the tumor cells increased progressively (Fig 6 and Fig 22). The relationship between serum glucose of the host and the number of glucose carrier of the tumor cells can be observed in the plot of serum glucose versus the reciprocal of carrier number (Fig. 23). A best-fit linear plot was obtained by linear regression with the correlation coefficient greater than 0.918. Therefore we can conclude that the carrier number on tumor cells are closely related to serum glucose of the host and the change in the number of glucose carrier on the tumor cells may be caused by the decrease in serum glucose of the host.

It has been demonstrated that the number of glucose carrier on cultured chick embryo fibroblasts is closely regulated by the level of glucose in culture medium. It was suggested that glucose acts as a "repressor" for carrier synthesis (Amos, Musliner and Asdourian 1977). Therefore whether serum glucose has any effect on the number of glucose carrier on Ehrlich ascites tumor cells *in vivo* was studied.

Fig. 23 The relationship between serum glucose of the host and the number of glucose carriers (Bo) in the tumor cells during tumor development. The reciprocal of Bo values of different days post transplantation are plotted versus the serum glucose of the mice on the corresponding days. The line was the best fit according to linear least squares analysis and with correlation coefficient greater than 0.918. Values are expressed as the mean and obtained from Fig. 6 and Fig. 22.

FIGURE 23



Insulin and streptozotocin were injected into different groups of mice to produce hypoglycemia and hyperglycemia respectively. The mice were then inoculated with Ehrlich ascites tumor cells. The serum glucose levels of the mice were examined during tumor development. As shown in Fig. 24, serum glucose of insulin-treated mice was lower than that of untreated mice while the serum glucose levels of streptozotocin-induced diabetic mice were much higher than that of untreated mice.

Injection of insulin to the streptozotocin-induced diabetic mice can abolish the hyperglycemia and restore the serum glucose level of streptozotocin-treated mice to normal range.

On day 7 post implantation, all groups of mice were killed and their tumor cells were harvested by exhaustive drainage. The tumor cell number was determined with haemocytometric cell counting. Table 3 shows the tumor size of the four different groups, the tumor size of control group is the largest. Insulin treatment has little effect on tumor growth while the injection of streptozotocin can cause a drastic and statistically significant reduction ($p < 0.01$) by about 80% in the tumor size. Injection of insulin to the streptozotocin-induced diabetic mice can only partially restore the tumor size of the mice.

Table 4 shows the number (Bo) and the dissociation constant (K_d) of glucose carriers on Ehrlich ascites tumor cells harvested from different groups of mice bearing 7 day-old tumors.

Fig. 24 Concentration of glucose in mouse serum during tumor development. Serum glucose of (● - ●) untreated mice, (☆ - ☆) insulin induced hypoglycemia mice, (○ - ○) streptozotocin-induced diabetic mice and (◇ - ◇) streptozotocin-induced diabetic mice with insulin treatment were determined every 2 days after tumor inoculation. ★ indicates day of injection of streptozotocin (200 mg/kg body weight). ⬍ indicates the days of injection of insulin (a - 4 IU/mouse, b - 3 IU/mouse, c - 2 IU/mouse and d - 1 IU/mouse). ▲ indicates the day of inoculation of tumor cells. Broken lines are the estimation of changes in serum glucose of different groups relative to untreated group. Values are presented as the mean ± S.E.M. for two separate experiments.

FIGURE 24

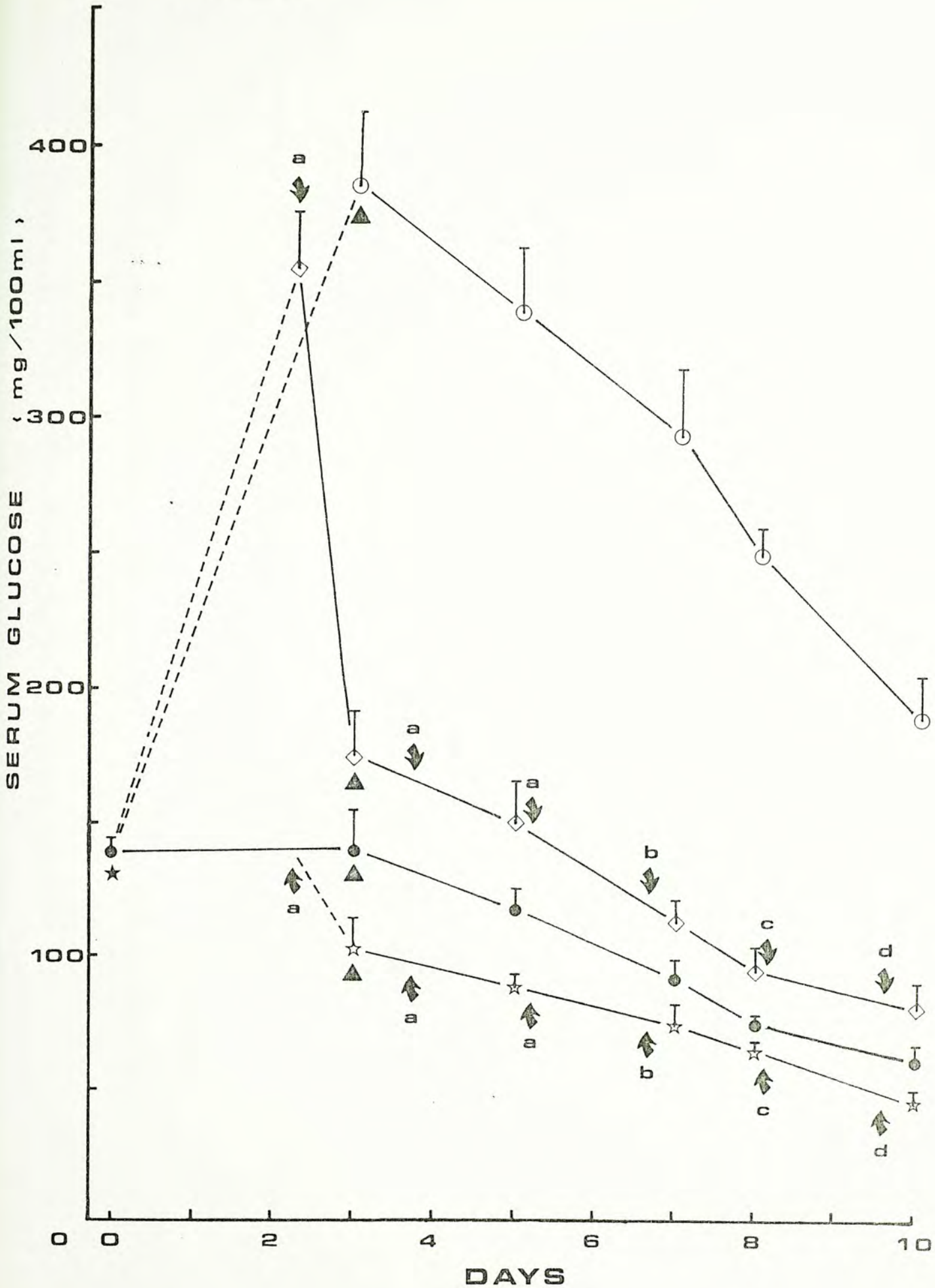

SERUM GLUCOSE (mg/100ml)
400
300
200
100
0
0
2
4
6
8
10
DAYS
a
a
a
a
a
b
b
c
c
d
d

TABLE 3. EFFECT OF STREPTOZOTOCIN AND INSULIN TREATMENT ON THE TUMOR SIZE OF EHRLICH ASCITES TUMOR BEARING MICE[a]

Groups	Treatment	Tumor size of 7 day-old tumors (10^8 cell/mouse)	Percentage of reduction
I	Nil	11.5 ± 0.4	0
II	Insulin injection every 36 hours	9.2 ± 0.5	20% ($p > 0.1$)
III	Streptozotocin-induced diabetes with insulin treatment	6.6 ± 0.6	43% ($p < 0.05$)
IV	Streptozotocin-induced diabetes	2.2 ± 0.5	81% ($p < 0.01$)

a. The treatment of streptozotocin and insulin to the tumor bearing mice was performed as described in the text. The values are presented as mean ± S.E.M. for 2 separate experiments.

TABLE 4. *IN VIVO* EFFECT OF STREPTOZOTOCIN AND INSULIN TREATMENT ON THE APPARENT DISSOCIATION CONSTANT (K_d) AND THE NUMBER (Bo) OF GLUCOSE CARRIER ON EHRLICH ASCITES TUMOR CELLS[a]

Groups	Treatment	Bo (pmol/10^7 cell)	K_d (10^{-7} M)	% change in Bo
I	Nil	211.7 ± 5.0	2.52 ± 0.08	0
II	Insulin injection every 36 hours	259.7 ± 6.2	2.76 ± 0.06	+ 23*
III	Streptozotocin-induced diabetes with insulin treatment	177.4 ± 4.6	2.28 ± 0.06	- 16*
IV	Streptozotocin-induced diabetes	117.3 ± 3.5	2.60 ± 0.0	- 45*

a. Treatment of streptozotocin and insulin to the tumor bearing mice was performed as described in the text. The values are presented as mean ± S.E.M. for triplicate determinations of 2 separate experiments.

* p value for the significance of difference < 0.001.

Bo in Ehrlich ascites tumor cells harvested from untreated mice is 212 pmol/10^7 cell. Ehrlich ascites tumor cells harvested from the insulin-induced hypoglycemic mice showed Bo of 260 pmol/10^7 cell. The difference between these two values is statistically significant ($p < 0.001$). By contrast Bo of tumor cells harvested from the streptozotocin-induced diabetic mice significantly decreases to 117 pmol/10^7 cell with $p < 0.001$ as compared with the value in those cells harvested from untreated mice. Injection of insulin to this diabetic group can partially restore the serum glucose to normal range as well as the Bo to 177 pmol/10^7 cell in the tumor cells.

The relationship between serum glucose of the tumor bearing mice and the number of glucose carriers (Bo) in the tumor cell can be further confirmed in Fig. 25. In the figure, the reciprocal of Bo for 7 day-old tumors harvested from the four different groups of mice was plotted versus the serum glucose on day 7 post tumor transplantation of the corresponding group of mice. A best-fit straight line was obtained by linear regression method with correlation coefficient about 0.990. It is more conclusive that the glucose carriers number (Bo) on tumor cells are closely related to serum glucose of the hosts and the change in Bo on the tumor cells may be caused by the changes in serum glucose of the host.

The K_d of the glucose carrier on the tumor cells remained

Fig. 25 The relationship between serum glucose of the hosts treated with insulin or streptozotocin and the number of glucose carrier (Bo) in Ehrlich ascites tumor cells harvested from the treated and untreated mice. The reciprocal of Bo values for different groups (Table 4) are plotted versus the serum glucose of mice bearing 7 day-old tumors (Fig. 24) of the corresponding groups. The line is the best fit according to linear least squares analysis and with correlation coefficient about 0.990. Values are expressed as the mean and determined from Fig. 24 and Table 4.

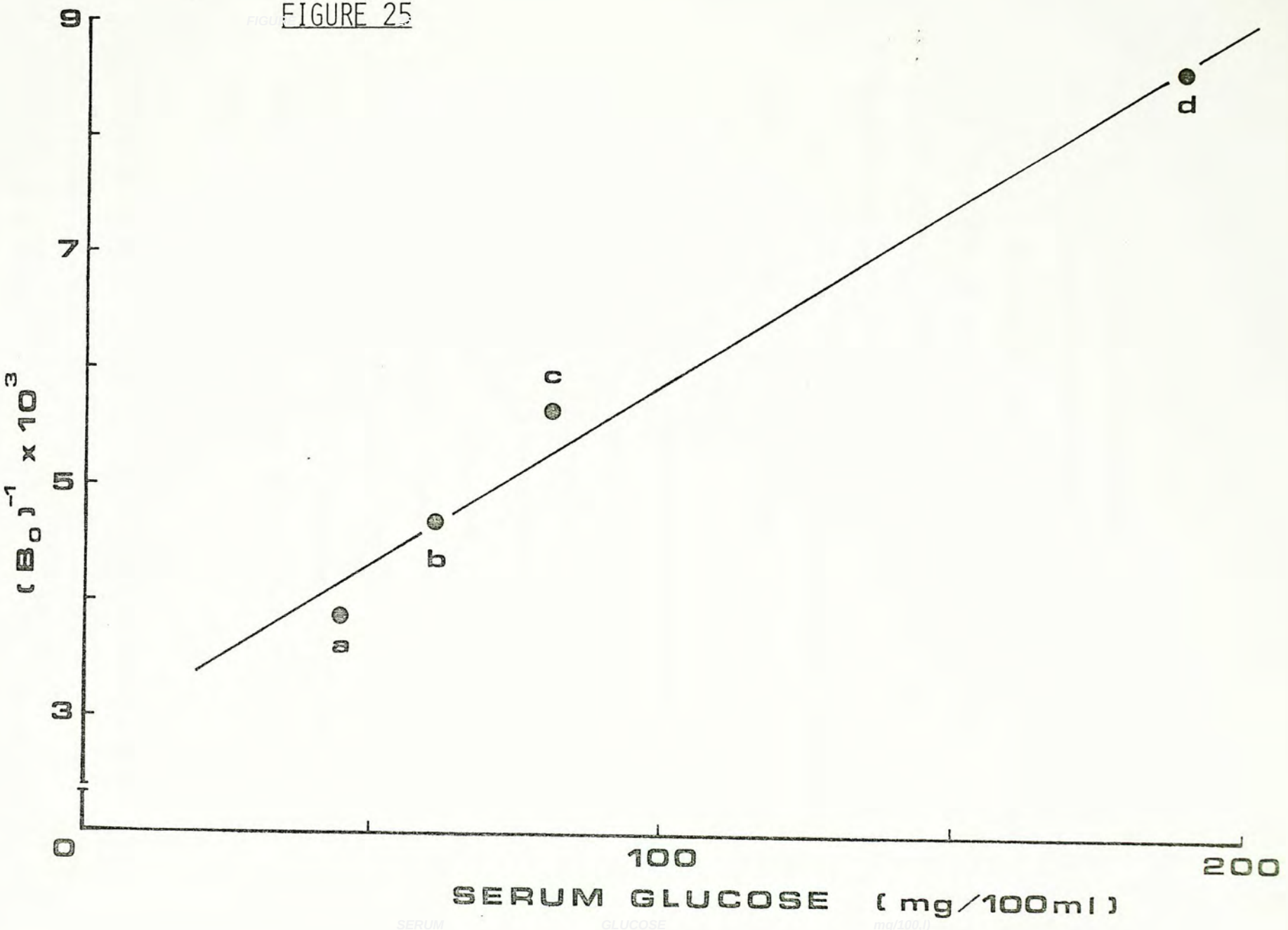
a. insulin-treated group;

b. untreated mice;

c. streptozotocin-induced diabetes with insulin treatment; and

d. streptozotocin-induced diabetic mice.

FIGURE 25



unchanged in both four groups indicating that the nature of the glucose carriers may not be affected by streptozotocin or insulin. Base on the above results, it can be interpreted that the number of glucose carrier (Bo) in tumor cells is closely related to the serum glucose levels of their hosts. However, the direct effect of insulin on Bo may not be ruled out since injection of insulin to the mice caused an increase in Bo, tumor cells harvested from mice with depletion of insulin by streptozotocin caused a decrease in Bo and injection of insulin to streptozotocin-induced diabetic (insulin deficient) mice can partially restore their Bo value. Furthermore, it has been shown that insulin can increase glucose carriers on plasma membrane pool and stimulate the translocation of glucose carriers from microsomal pool to plasma membrane in adipocytes (Karnieli *et al* 1981; Sogin and Hinkle 1980; Suzuki and Kono 1980). To differentiate whether Bo on Ehrlich ascites tumor cells is affected by glucose or insulin, direct effect of insulin on Bo was examined.

VII. Effect of hormones on the number of glucose carrier (Bo) in Ehrlich ascites tumor cells

Table 5 shows the number (Bo) and apparent dissociation constant (K_d) of glucose carriers on Ehrlich ascites tumor cells after incubation with different hormones. The incubation of Ehrlich ascites tumor cells in incubation buffer at

37° C for 60 minutes (group II) has no effect on Bo and K_d comparing with group I ($p > 0.1$). The addition of insulin at a low concentration (group III, 2 μg/ml, i.e. 0.05 unit/ml) or a high concentration (group IV, 20 μg/ml, i.e. 0.5 unit/ml) in the incubation buffer and incubating for 60 minutes does not affect Bo. However, addition of glucagon (group V, 5×10^{-3} mg/ml) caused a significant reduction comparing with group II ($p < 0.001$) in Bo by about 35%. Similarly, the addition of corticosterone (group VI, 25×10^{-3} mg/ml) and its metabolite, cortisone (group VII, 25×10^{-3} mg/ml), also caused significant reduction comparing with group II ($p < 0.001$) in Bo by about 34% and 36% respectively. The K_d values of the glucose carriers on Ehrlich ascites tumor cells are not affected by the addition of various hormones throughout incubation.

According to the results shown above, the direct effect of insulin on Bo in Ehrlich ascites tumor cells may be insignificant. In order to further investigate the effect of extracellular glucose level on Bo, the tumor cells were incubated with different glucose concentration in medium *in vitro* to see whether there is any change in Bo after the incubation.

TABLE 5. THE EFFECT OF DIFFERENT HORMONES ON THE APPARENT DISSOCIATION CONSTANT (K_d) AND THE NUMBER (Bo) OF GLUCOSE CARRIER ON EHRLICH ASCITES TUMOR CELLS AFTER *IN VITRO* INCUBATION[a]

Groups	Hormones added	Concentration of the hormones	Bo (pmol/10^7 cell)	K_d (10^{-7} M)	% change in Bo
I[b]	Nil	-	216.0 ± 8.7	2.72 ± 0.09	-[c]
II[d]	Nil	-	229.7 ± 7.4	2.60 ± 0.06	0
III	Insulin	20 µg/ml	221.3 ± 2.7	2.67 ± 0.09	-4 ($P > 0.1$)
IV	Insulin	2 µg/ml	220.2 ± 6.7	2.43 ± 0.11	-4 ($p > 0.1$)
V	Glucagon	5 µg/ml	149.5 ± 5.5	2.52 ± 0.08	-35 ($p < 0.001$)
VI	Corticosterone	25 µg/ml	152.7 ± 6.4	2.23 ± 0.09	-34 ($p < 0.001$)
VII	Cortisone	25 µg/ml	146.7 ± 6.0	2.33 ± 0.09	-36 ($p < 0.001$)

a. The incubation of various hormones with 7 day-old tumor cells was performed as described in the text. The values are presented as mean ± S.E.M. for triplicate sample determinations of 2 separate experiments.

b. Without incubation at 37° C

c. Not compared

d. With incubation at 37° C for 60 minutes

VIII. The effect of glucose in incubation medium on the number of glucose carriers in Ehrlich ascites tumor cells *in vitro*

Ehrlich ascites tumor cells were incubated in RPMI 1640 medium supplemented with glucose either at 5.5 mg/ml or 1.9 mg/ml. Aliquots of tumor cell suspension were taken out at different time intervals. The supernatants of cell suspension were tested for glucose concentration and the tumor cells were harvested and washed twice with PBS, and the washed cells were then tested for glucose carrier content. Fig. 26 shows the changes in glucose concentration in the medium and the Bo in the tumor cells throughout the incubation in low glucose medium (1.9 mg/ml). Curve A shows the glucose concentration in the medium at different time points. The glucose concentration fell from 1.88 mg/ml at time zero to zero concentration at 8 hours post incubation and remained at the value of zero concentration afterwards. Curve B shows the changes in Bo throughout the incubation. Bo decreased by about 12% in the first 2 hours of incubation. In the period of 2 hours to 8 hours post incubation, Bo increased to the original value and remained unchanged until 14 hours post incubation. However in the period of 14 hours to 26 hours post incubation, Bo increased drastically. The values of Bo at 20 hours and 26 hours post incubation were 276 and 302 pmol/10^7 cell respectively. These increments were about 28% ($p < 0.005$) and 40% ($p < 0.005$) respectively comparing with the Bo of 215 pmol/10^7 cell at time zero. Fig. 27 shows the changes in glucose concentration in the medium and Bo in tumor cells throughout the incubation in high glucose medium (5.5 mg/ml).

Fig. 26 The effect of glucose concentration of the medium on the number of glucose carriers (Bo) in Ehrlich ascites tumor cells incubated in the medium. Curve A shows the change in glucose concentration (★-★) in the low glucose medium (1.9 mg/ml). Curve B (●-●) shows the change in Bo in the tumor cells. Bo value increase by about 28% ($p < 0.005$) and 40% ($p < 0.005$) for 20 and 26 hours post incubation respectively comparing with the value of the beginning of incubation. Values are presented as the mean ± S.E.M. for triplicate determinations.

FIGURE 26

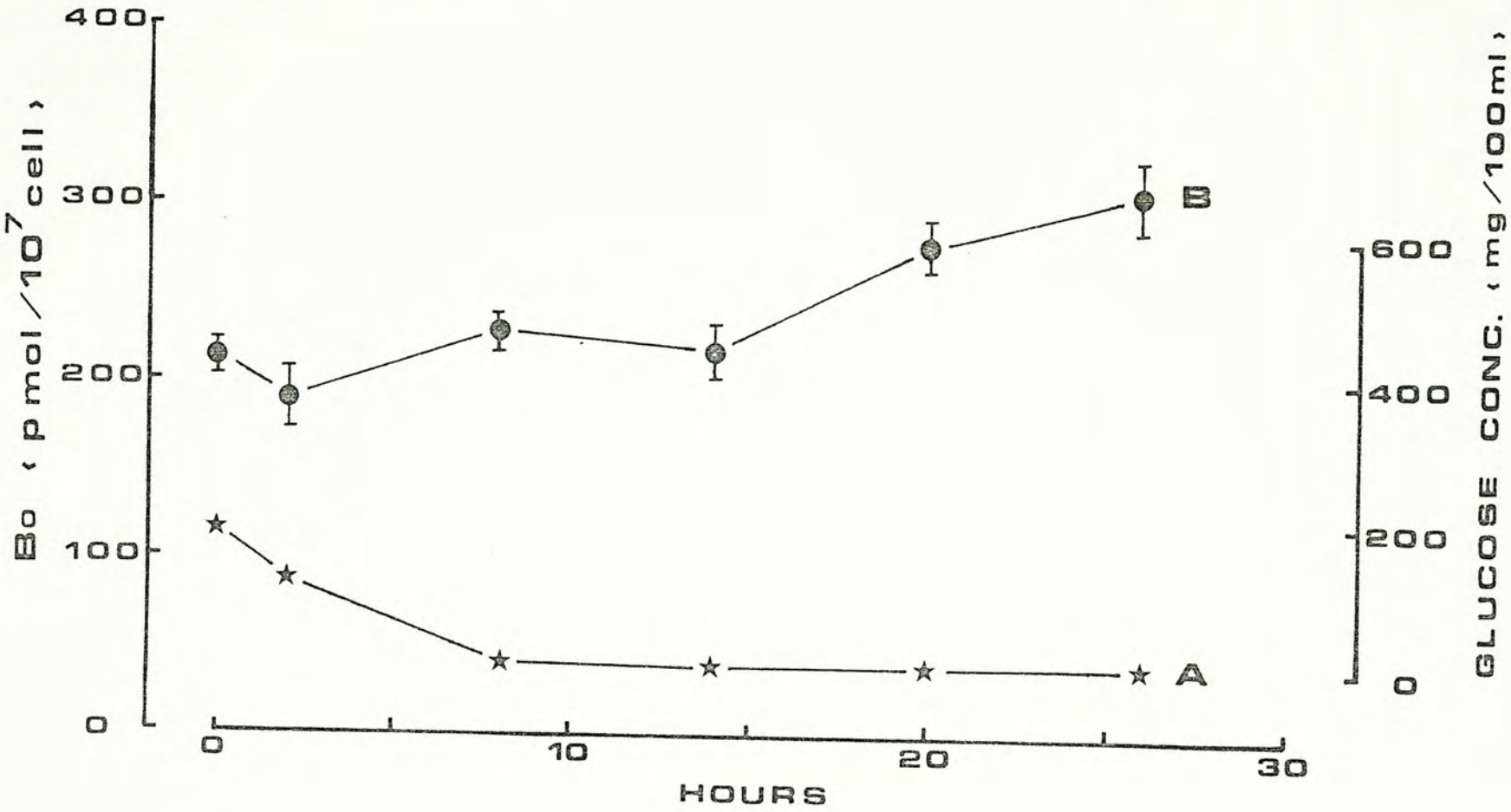

400
300
200
100
0
Bo ‹ p mol/10⁷ cell ›
B
A
0
10
20
30
HOURS
600
400
200
0
GLUCOSE CONC. ‹ mg/100ml ›

Fig. 27 The effect of glucose concentration of the medium on the number of glucose carriers (Bo) in Ehrlich ascites tumor cells incubated in the medium. Curve A shows the change in glucose concentration (★ - ★) in the high glucose medium (5.5 mg/ml). Curve B (● - ●) shows the changes in Bo in the tumor cells. Bo values decrease by about 32% ($p < 0.001$) in the first 8 hours of incubation comparing with the value of the beginning of incubation. Values are presented as the mean ± S.E.M. for triplicate determintations.

FIGURE 27

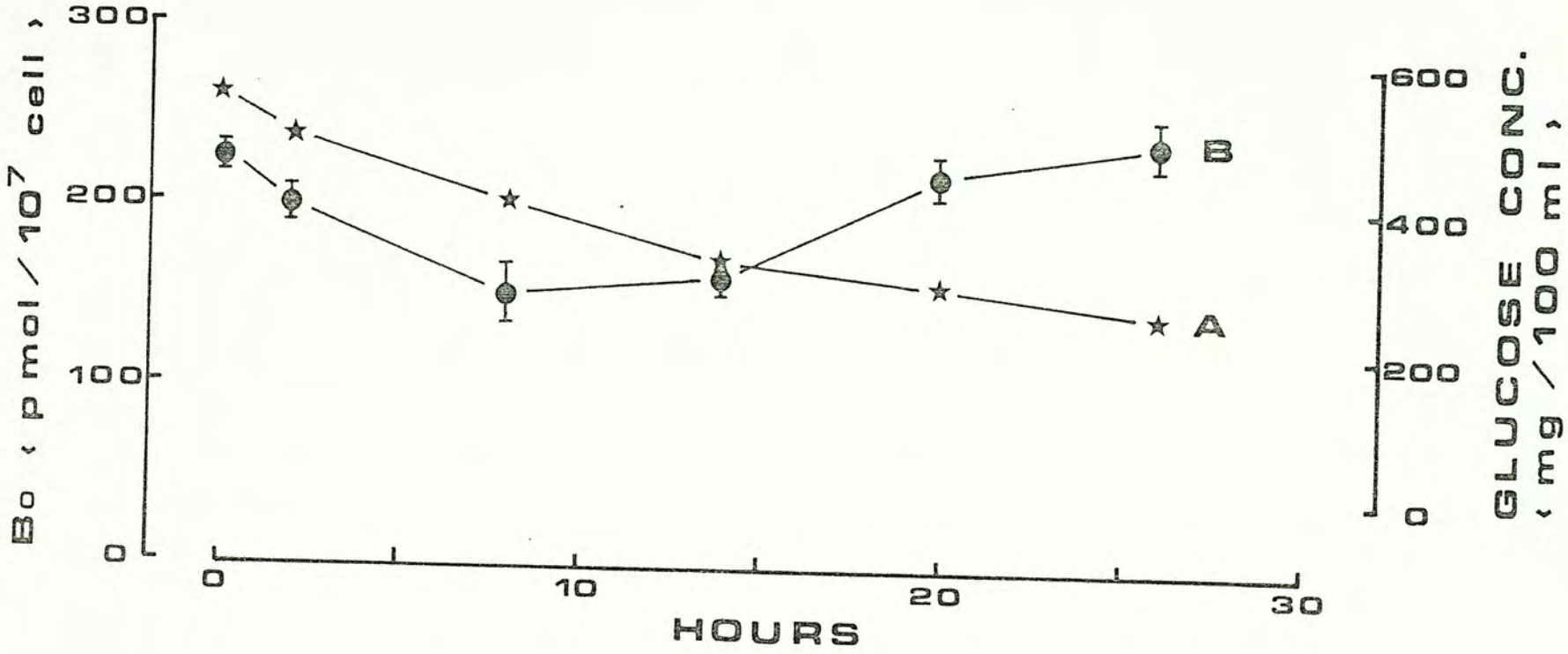

Bo ‹ p mol / 10^7 cell ›
300
200
100
0
0
10
20
30
HOURS
B
A
600
400
200
0
GLUCOSE CONC.
‹ mg / 100 ml ›

TABLE 6. THE APPARENT DISSOCIATION CONSTANT (K_d) OF GLUCOSE CARRIER ON EHRLICH ASCITES TUMOR CELLS AFTER INCUBATION IN RPMI 1640 MEDIUM *IN VITRO*[a]

Time of incubation (hours)	Apparent dissociation constant (K_d) (10^{-7} M)	
	High glucose medium group	Low glucose medium group
0	2.67 ± 0.09	2.63 ± 0.12
2	2.40 ± 0.10	2.63 ± 0.09
8	2.37 ± 0.12	2.23 ± 0.07
14	2.47 ± 0.18	2.57 ± 0.09
20	2.77 ± 0.07	2.73 ± 0.03
26	2.73 ± 0.12	2.70 ± 0.06

a. The tumor cells were incubated in low glucose (1.9 mg/ml) and high glucose (5.5 mg/ml) medium. The incubation was performed as described in the text. The K_d value for these two groups are apparently remained unchanged. The p values for the significance of difference for low glucose medium group is > 0.05 and that for high glucose medium group is > 0.1. Values are presented as mean ± S.E.M. for triplicate determinations.

Curve A shows the glucose concentration in the medium at different time points. The glucose concentration fell progressively from 5.53 mg/ml at the beginning to 2.53 mg/ml at 26 hours post incubation. Curve B shows the changes in Bo throughout incubation. Bo decreased by about 32% in the first 8 hours and afterwards it began to rise gradually and reached the original value at about 22 hours post incubation. Then it remained relatively unchanged until 26 hours post incubation. Table 6 shows the values of apparent dissociation constant (K_d) of the glucose carriers on Ehrlich ascites tumor cells at different time points. There are no significant change in K_d for high glucose group ($p > 0.1$) and low glucose group ($p > 0.05$) throughout the incubation period, furthermore, K_d value is also not affected by the glucose concentration of the medium in which the tumor cells were incubated.

IX. *In vivo* effect of methotrexate (MTX) on 2-deoxy-D-glucose uptake and the glucose carrier on Ehrlich ascites tumor cells

The effect of methotrexate (MTX) on the uptake of 2-deoxy-D-glucose and glucose carrier on Ehrlich ascites tumor cells was studied with 7 day-old tumor cells. Administration of 0.4 mg MTX per Kg body weight can decrease the tumor size of tumor bearing mice (Table 7). Earlier administration of MTX (on days 2, 4 and 6) caused a greater reduction (by about 53%) in tumor size than that (by about 31%) caused by later administration (on days 4, 5 and 6). Administration of MTX on days 4, 5 and 6 post transplantation reduced the Vmax of the uptake process

significantly ($p < 0.005$) by about 25% (Table 8). This decrease could be shown to be due principally if not exclusively to a reduction in Bo per cell, as evidenced by the 25% reduction in the Bo value in MTX-treated cells when compared with that of untreated cells (Table 9). The apparent K_M value for the hexose uptake process was unaffected by MTX; correspondingly, no significant difference was obtained for the apparent dissociation constant (K_d) for the glucose carriers ($p > 0.1$) (Table 8 and Table 9). Table 8 also shows that MTX administratered on days 2, 4 and 6 post transplantation, which was more effective in reducing tumor size, brought about a more pronounced drop in the rate of glucose uptake, and a bigger reduction in the Bo (Table 9). The magnitude of changes in the later two parameters was again comparable, 35% versus 40%. Similarly, the values of K_M and K_d were not affected by the administration of MTX ($p > 0.1$) (Table 8 and Table 9). Furthermore, the values of K_M and K_d were not affected by the time intervals of MTX administration.

TABLE 7. THE EFFECT OF METHOTREXATE (MTX) ON THE TUMOR SIZE OF THE TUMOR BEARING MICE *IN VIVO*[a]

Groups	Dosage of MTX (mg/Kg body wt/injection)	Days of MTX administration post implantation	Tumor size (10^8 cell/mouse)	% reduction
Control	0	0	10.8 ± 0.4	0
A	0.4	2, 4, 6	5.1 ± 0.2	53 ($p < 0.01$)
B	0.4	4, 5, 6	7.4 ± 0.3	31 ($p < 0.05$)

a. The values represent the tumor size of mice on day 7 post tumor transplantation and are expressed as mean ± S.E.M. for 3 separate experiments.

TABLE 8. THE EFFECT OF METHOTREXATE (MTX) ON MAXIMAL UPTAKE RATE (Vmax) AND APPARENT HALF-SATURATION CONSTANT (K_M) OF THE PROCESS OF 2-DEOXY-D-GLUCOSE UPTAKE BY EHRLICH ASCITES TUMOR CELLS[a]

Groups	2-deoxy-D-glucose uptake		% reduction in Vmax
	Vmax (nmol/min/5 x 10^7 cell)	K_M (mM)	
Control	157.3 ± 5.8	0.64 ± 0.07	0
A[b]	102.3 ± 2.8	0.52 ± 0.03	35 ($p < 0.001$)
B[c]	117.7 ± 4.3	0.58 ± 0.03	25 ($p < 0.005$)

a. The uptake of 2-deoxy-D-glucose was measured as described in the text using the cells harvested from treated and untreated 7 day-old tumors. Values are expressed as mean ± S.E.M. of triplicate sample determinations.

b. Mice were administered with 0.4 mg/Kg body wt MTX on days 2, 4, 6 post tumor inoculation.

c. Mice were administered with 0.4 mg/Kg body wt MTX on days 4, 5, 6 post tumor inoculation.

TABLE 9. THE EFFECT OF METHOTREXATE (MTX) ON APPARENT DISSOCIATION CONSTANT (K_d) AND THE NUMBER (Bo) OF GLUCOSE CARRIERS IN EHRLICH ASCITES TUMOR CELLS[a]

Groups	Glucose carrier		% reduction in Bo
	Bo (pmol/10^7 cell)	K_d (10^{-7} M)	
Control	221.3 ± 7.9	2.48 ± 0.07	0
A[b]	130.0 ± 9.8	2.20 ± 0.08	41 ($p < 0.001$)
B[c]	162.8 ± 7.2	2.17 ± 0.11	26 ($p < 0.001$)

a. The glucose carriers were measured as described in the text using cells harvested from treated or untreated 7 day-old tumors. Bo and K_d were obtained from Scatchard analysis of the difference in binding in the absence and in the presence of 500 mM of D-glucose. Values are expressed as mean ± S.E.M. of triplicate sample determinations from 2 separate experiments.

b. Mice were administered with 0.4 mg/Kg body wt MTX on days 2, 4, 6 post tumor inoculation.

c. Mice were administered with 0.4 mg/Kg body wt MTX on days 4, 5, 6 post tumor inoculation.

DISCUSSION

I. Changes in glucose uptake by Ehrlich ascites tumor cells during tumor development *in vivo*

The present study showed that the rate of glucose uptake by Ehrlich asciter tumor cells increased progressively as the tumor developed (Fig. 4). When present at high enough concentration, glucose can enter cells at a significant rate by simple diffusion (unsaturable diffusion) (Fig. 3B; Renner, Plagemann and Bernlohr 1972; Plagemann and Richey 1974). The negligibly low level of glucose in the ascites fluid (Fig. 22), however, would require that, *in vivo*, Ehrlich ascites tumor cells obtained glucose through a facilitated transport process and this is consistent with the idea of Fishman and Bailey (1974). Such a process has been shown to follow simple Michaelis-Menten kinetics and may adequately be described by the kinetic parameters, Vmax and K_M (Plagemann and Richey 1974; Kaminskas 1979). The data presented in Fig. 4 showed that the Vmax for glucose uptake increased continuously with tumor growth but the K_M value remained constant throughout.

Methotrexate, an antimetabolite demonstrated to inhibit glucose consumption by cultured Ehrlich ascites tumor cells (Kaminskas and Nussey 1978) was also seen in the present study to decrease glucose uptake by lowering the Vmax value without appreciably affecting the K_M value (Table 3, group A and B). Increased glucose and nucleosides uptake has been accompany phytohaemagglutinin-stimulated proliferation of cultured bovine lymphocytes (Peters and Hawn 1971 a and b) as well as

the virus-induced neoplastic transformation of several avian and mammalian cell lines (see Hatanaka 1974 for review) including Rous sarcoma virus/chick embryo fibroblasts (Martin *et al* 1971; Hatanaka and Hanafusa 1970; Lee and Lipmann 1977; Lawrence and Jullien 1980), polyoma virus/mouse BHK cells (Isselbacher 1972) and hamster pseudotype sarcoma virus/hamter embryo cells (Hatanaka, Gilden and Kelloff 1971). Normal cell lines including mouse and chick cells in culture, on the other hand, exhibited reduced transport rates when they attained confluency (Bose and Zlotnick 1973; Rubin 1972), as did rat 3T3-L1 fibroblasts upon conversion to adipocytes with dexamethasone/1-methyl-3-isobutylxanthine treatment (Resh 1982). The changed glucose transport rate in each case was shown to be the result of changes in values of Vmax rather than K_M. In this respect, glucose transport in these experimental system resembles that in Ehrlich ascites tumor cells. Transport rates changed because of changes in the number and/or operational efficiency of transport sites; the affinity between glucose and its transport component was not affected, at least not in the aforementioned circumstances.

II. Characteristics of glucose-sensitive cytochalasin B binding sites on Ehrlich ascites tumor cells

In nearly all eukaryotic cells studied, glucose transport occurs as a carrier-mediated process (see Jones and Nickson 1981 for review). Cytochalasin B inhibits this process and a set of high-affinity glucose inhibitable binding sites for

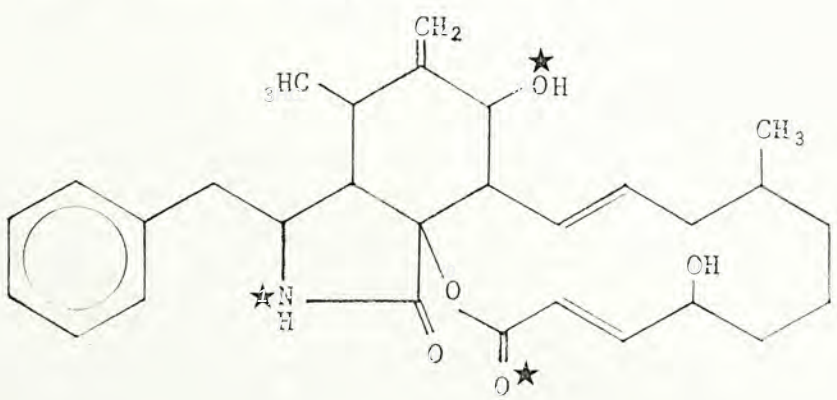
cytochalasin B has been tentatively identified to be the glucose transporter (Karnieli *et al* 1981; Cuppoletti, Mayhew and Jung 1981; Sogin and Jung 1981; Sogin and Hinkle 1980a; Suzuki and Kono 1980; Jung and Rampal 1977). Studies performed on this glucose-reversible cytochalasin B binding activity in cell systems as divergent as human erythrocytes, Novikoff rat hepatoma cells and barnacle muscle cells (Cuppoletti, Mayhew Jung 1981; Renner, Plagemann and Bernlohr 1972; Sogin and Hinkle 1980a; Jung and Rampal 1977; Baker and Carrnthers 1980) revealed that they share a number of common characteristics : (1) a high affinity for cytochalasin B (K_d 1-6 x 10^{-7} M); (2) stereospecific inhibition by sugar; thus D-glucose, 3-O-methyl-D-glucose, 2-deoxy-D-gluocse, D-galactose and D-mannose inhibit binding; L-glucose and L-fucose are without effect; and (3) sensitivity to phloretin and diethylstilbestrol.

These properties are also examined in the present studies with Ehrlich ascites tumor cells (Fig. 5A and 5B, Fig. 8A and 8B, Fig. 9A and 9B, Fig. 10A and 10B, Fig. 11A and 11B, Fig. 13A and 13B, Fig. 14A and 14B, Fig. 15, Fig. 17 and Table 2). It is found that the dimer of glucose, maltose, can also partially displace cytochalasin B from its binding sites. However, other disaccharides like lactose, dimer of galactose and glucose, and sucrose, dimer of fructose and glucose, cannot displace any cytochalasin B from its binding sites. It seems that the existence of other saccharides in the glucose-containing disaccharides will hinder the binding of these disaccharides to the glucose-sensitive cytochalasin B binding

sites. On the other hand, monosaccharides like 3-O-methyl-D-glucose and 2-deoxy-D-glucose, which are derivatives of D-glucose, can completedly displace while D-mannose and D-galactose can only partially displace cytochalasin B from its binding sites. By contrast, fructose, arabinose cannot undergo this displacemant. The binding sites seem to be stereospecific that no binding can be observed in L-glucose. Glucosamine, with an amine group on glucose molecule, cannot bind to the glucose-sensitive cytochalasin B binding sites on Ehrlich ascites tumor cells. This observation is in contrast with that reported in other cell lines (Plagemann *et al* 1981; Hatanaka 1974).

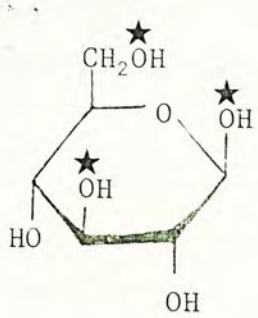
Recently, Griffin, Rampal and Jung (1982) suggested that cytochalasin B bound to the glucose carrier on human erythrocytes through hydrogen bond at N2 (donor), O7 (acceptor), and O23 (acceptor) analogous to O6, O3 and O1 respectively on β-D-glucose. The hydrophobic region from C13 to C19 also essential in binding to carrier molecules and appears to act as an anchor in a hydrophobic domains of the glucose carrier. The structures of cytochalasin B and various sugars are shown below and the indicated area (★) is the group suggested to be responsible for the binding.

The structure of cytochalasin B :

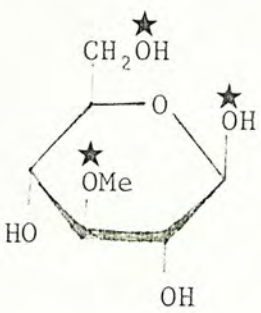


Structures of the sugars which can bind on glucose-sensitive cytochalasin B binding sites :

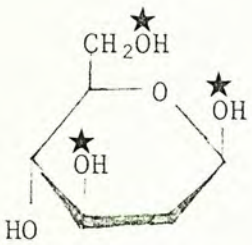
β-D-glucose



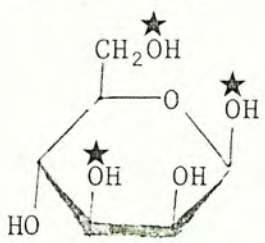
3-O-methyl-β-D-glucose



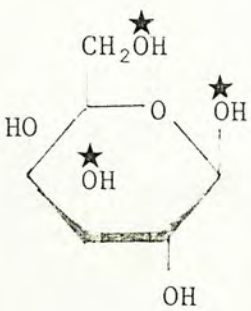
2-deoxy-β-D-glucose



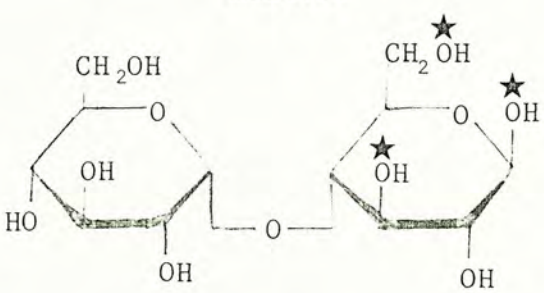
β-D-mannose



β-D-galactose

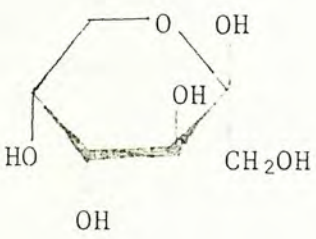


maltose

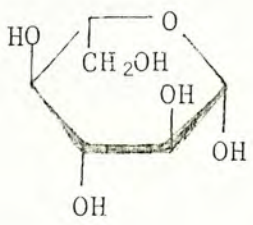


Structures of the sugars which cannot bind on glucose-sensitive cytichalasin B binding sites :

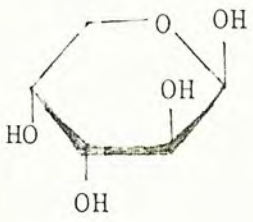
β-D-fructose



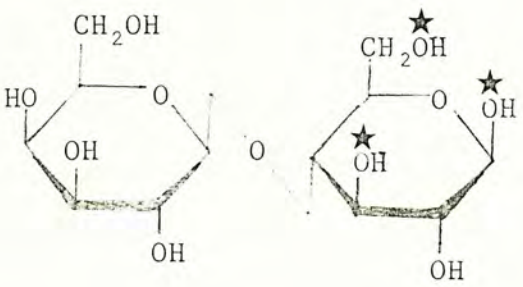
β-L-glucose



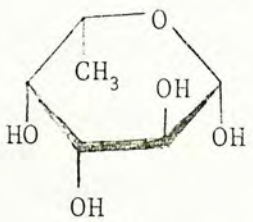
β-D-arabinose



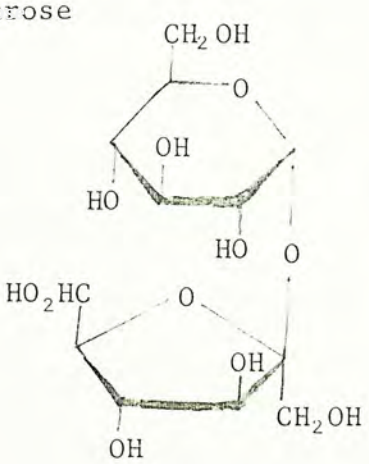
lactose

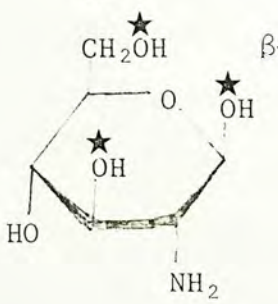


β-L-fucose



sucrose





β-D-glucosamine

According to the proposal of Griffin, Rampal and Jung (1982), all monosaccharides and disaccharides bearing the same configuration of O6, O3 and O1 as in β-D-glucose like β-D-mannose, β-D-galactose, 2-deoxy-β-D-glucose, 3-O-methyl-β-D-glucose, β-D-glucosamine, maltose and lactose can bind on glucose-sensitive cytochalasin B binding sites and displace cytochalasin B from these sites. While other sugars with different configuration of O6, O3 and O1 as β-D-glucose like D-fructose, D-arabinose, L-fucose, L-glucose and sucrose cannot bind on these glucose-sensitive cytochalasin B binding sites.

This postulation can only be confirmed in parts in our experiments. D-fructose, D-arabinose, L-fucose, L-glucose and sucrose cannot bind on glucose-sensitive cytochalasin B binding sites on Ehrlich ascites tumor cells as expected. However, the present study showed that D-mannose and D-galactose can only partially displace cytochalasin B from its glucose-sensitive binding sites and even no displacement could be observed in lactose and glucosamine. The glucose uptake inhibitors, plioretin and diethylstilbestrol can also displace cytochalasin B from its glucose-sensitive binding sites, our results showed that they might bind to the glucose-sensitive cytochalasin B binding sites on Ehrlich ascites tumor cells and compete with cytichalasin B. However, Jennings and Solomon (1976) suggested that these uptake inhibitors may affect the dipole potential at membrane surface. The nature of substrate specificity of the glucose-sensitive cytichalasin B binding sites on Ehrlich ascites tumor cells

and other cell lines remains not known and awaits further investigations.

III. Identification of glucose-sensitive cytichalasin B binding sites as glucose carriers on Ehrlich ascites tumor cells

There are three experimental evidences which strongly suggest that the glucose-sensitive cytochalasin B binding sites are identical to the glucose carriers on Ehrlich ascites tumor cells. Firstly, it should be noted that in nearly all eukaryotic cells studied, glucose-sensitive cytochalasin B binding sites on the cells are believed to be identical to the glucose carriers on these cells (see Jones and Nickson 1981 for review). Both the three common characteristics of the glucose carriers in all other cell lines studied, namely, high affinity for cytochalasin B binding, stereospecificity for D-form of sugars and sensitivity to glucose uptake inhibitors, has been shown to be shared by the glucose-sensitive cytochalasin B binding sites on Ehrlich ascites tumor cells. The structure of these sites on Ehrlich ascites tumor cells may be very close to those of other cells or they may even share a certain degree of common structure with those on other cell lines.

Secondly, the increase in number of putative glucose-sensitive cytochalasin B binding sites sufficiently accounted for the elevated rate of glucose uptake associated with tumor development. Fig. 4 shows that between day 2 and day 9 post transplantation, the Vmax value for glucose uptake process increased approximately 3.3 fold. During the same period,

the maximal number of glucose-sensitive cytochalasin B binding sites increased 3.4 fold (Fig. 6). Furthermore, the increase in glucose uptake rate progressively during tumor development was well correlated with the increase in glucose-sensitive cytochalasin B binding sites on Ehrlich ascites tumor cells during the same period (Fig. 7).

Thirdly, it may be seen that methotrexate suppressed the uptake of 2-deoxy-D-glucose in 7 day-old tumor cells to the same extent as glucose-sensitive cytochalasin B binding was inhibited (Table 8 and Table 9). Furthermore, the fact that methotrexate was without effect on the K_d for the cytochalasin B binding (Table 9) corroborates our earlier assertion that qualitative changes in the transport process are not related to substrate binding. It may therefore be concluded that changes in glucose uptake by Ehrlich ascites tumor cells are principally the result of changes in the number of transport carriers. While alteration in the turnover rate of glucose carriers cannot be absolutely ruled out in the present study, its contribution to the overall transport process is at best minimal. A similar conclusion has been reached by Wardzala *et al* (1978) for the effect of insulin on glucose transport in rat adipocytes.

If the postulation that this class of cytochalasin B binding sites are identical to the glucose carrier is valid, the data presented in Fig. 6 may be interpreted to mean that as the Ehrlich ascites tumor cells grew, the number of glucose carrier increased and thus glucose uptake increased.

The high density of glucose carriers on Ehrlich ascites

tumor cells is remarkable and unique among other animal cells in its high content of this site. In 7 day-old cells, the maximal binding of 220 pmol/10^7 cell (Table 9) translates to 1.3×10^7 carrier molecules/cell. This is in close agreemant with the value estimated by Cuppoletti *et al* (Cuppoletti *et al* 1981) in cultured Ehrlich ascites tumor cells and is considerably higher (3-100 fold) than that reported for human erythrocytes, Hela cells and Novikoff rat hepatoma cells (Sogin and Hinkle 1980a; Lin, Santi and Spudich 1974; Plagemann, Graff and Wohllueter 1977). This observation may probably reflects the fact that the other cells are mainly dependent on respiration for the provision of metabolic energy (Freudenberg and Mager 1971; Plagemann and Erbe 1973; Schwartz and Johnson 1976), Ehrlich ascites tumor cells depend primarily on glycolysis and a high-capacity system for glucose uptake is essential for the maintenance of growth (Kaminskas and Nussey 1978). The continuous fall in serum glucose during tumor development (Fig. 22) is a reflection of this high demand for glucose and the efficiency of the glucose carrier on Ehrlich ascites tumor cells.

IV. Change in the number of glucose carriers in Ehrlich ascites tumor cells during tumor development

The increases in glucose uptake and the number of glucose carriers during tumor development is difficult to explain. When 7 day-old tumor cells possessing about 200 pmol/10^7 cell were inoculated into normal mice, a 4-fold decrement in the

number of glucose carriers was observed within 48 hours post transplantation (Fig. 6). The glucose concentration in normal mice peritonael fluid is 95 mg/100 ml (Nakamura and Hosoda 1968). Once the tumor cells were inoculated, glucose concentration in ascites fluid decreased to apparently zero within 48 hours post transplantation (Fig. 22). From this time on, tumor cells were under glucose starvation. There are a large body of evidences showing that glucose starvation results in an apparent enhancement of glucose uptake in chick embryo fibroblasts, hamster rodent cells, human HSWP cells and Hela cells *in vitro* (Martineau *et al* 1972; Kalcker and Ullrey 1973; Hatanaka 1973; Shaw and Amos 1973; Kletzien and Perdue 1975; Salter and Cook 1976; Christopher, Colby and Ullrey 1976; Muskiner, Chrousos and Amos 1977; Salter and Cook 1975; Venuta and rubin 1975). This transport enhancement is RNA transcription and protein synthesis dependent. Banjo and Perdue (1976) and Adams *et al* (1977) both could identify two membrane proteins whose rate of synthesis was accelerated when chick cells were starved of glucose and protein synthesis was also observed in Rous sarcoma virus-transformed chick cells (Weber 1973; Hatanaka 1974; Lee and Lipmann 1977; Lawrence and Julien 1980). The implication for the increase in glucose uptake during glucose starvation is that glucose transporters are involved. Moreover, restoration of glucose to the medium on starved cells leads to a reversal of the glucose uptake enhancement to control value. It seems reasonable to postulate that glucose carriers on the cells are regulated by the

extracellular glucose concentration. Low glucose concentration can trigger the synthesis of glucose carriers while high glucose concentration can suppress the synthesis of carrier molecules. Christopher and coworkers (Christopher *et al* 1976; Christopher, Colby and Ullrey 1976) proposed that regulation in glucose transport carrier involves the turnover of components of glucose uptake systems on the one hand and glucose carrier synthesis which may or may not be alternatively accelerated and restrained on the other. Amos *et al* (1977) showed that hexose deprivation stimulates glucose uptake 30-fold in chick embryo fibroblasts. They showed that external energy source provided by a "non-repressor" sugar (D-fructose, D-xylose) or by pyruvate is required to accomplish carreir synthesis in glucose starved state. Furthermore glucose acts as a repressor at a posttranscriptional step probable at the level of turnover of formed carrier. Since the potection of formed carrier in the absence of glucose and by inhibitors of protein synthesis even in the presence of glucose has encouraged conjecture that a protease is activated by a metabolic product of glucose that is analogous to a corepressor. The metabolite either activates the protease by direct interaction with it or alters the conformation of the carrier molecules to expose a critical region to protease attack. The regulation of carrier density in the membrane may be archieved entirely by carrier inactivation, the rate of which is a function of glucose concentration in the culture medium. If these repression and derepression hypothe[illegible] for [illegible]ose [illegible]ke attributed

by glucose carrier molecules also appeared in Ehrlich ascites tumor cells, the number of glucose carriers in Ehrlich ascites tumor cells changes during tumor development *in vivo* may also be due to the high glucose concentration in normal mice peritoneal fluid which causes repression in glucose carrier, and the absence of glucose in ascites fluid resulting derepression in glucose carrier.

V. Relationship between glucose concentration and glucose carriers on Ehrlich ascites tumor cells

The glucose presented in peritoneal fluid was deliveried from the systemic circulation. In normal mice, glucose concentration in peritoneal fluid is 95 mg/100 ml. When 7 day-old Ehrlich ascites tumor cells were exposed to this high concentration of glucose, their glucose carrier density might be under the regulation of repression and decreased by about 4-fold and glucose could enter the tumor cells by simple diffusion (unsaturable diffusion). Therefore decrease in the number of glucose carrier in Ehrlich ascites tumor cells when exposed to fresh peritoneal fluid within 48 hours post transplantation could be explained by external glucose regulation. However, Ehrlich ascites tumor cells metabolized glucose at a extremely high rate, as they proliferated in the peritoneal cavities of of the mice, glucose in ascites fluid was used up. Because of the glucose concentration gradient between systemic circulation and ascites fluid, serum glucose would be continuously deliveried into the peritoneal cavities of the Ehrlich ascites

tumor bearing mice, but once the glucose was flowed in, it was metabolized by the tumor cells. Therefore in ascites fluid, glucose concentration was essentially undetectably low. The serum glucose decreased progressively during tumor development, the delivery rate of serum glucose to ascites fluid would also decreased progressively within the same period since the decrease in glucose concentration gradient. However, tumor cells could proliferate and increase their cell number during tumor development. Therefore it is reasonable to suggest that under glucose starvation, Ehrlich ascites tumor cells should not only possess an active transport system with a high affinity for glucose but also possess the ability to increase its glucose carrier to take up glucose required for their proliferation during development. This may represent a useful adaptive capability of tumor cells. The apparent low K_M value (about 0.6 mM) of glucose uptake process in Ehrlich ascites tumor cells can take up glucose from ascites fluid in which glucose concentration is undetectably low (Fig. 22). Fig. 23 can clearly show that the number of glucose carriers in Ehrlich ascites tumor cells is well correlated with the level of serum glucose of the hosts.

In order to investigate the above postulation, insulin and streptozotocin were injected to produce hypoglycemia and hyperglycemia in tumor bearing mice. Streptozotocin can destroy pancreatic β-cells and deplete insulin in mice *in vivo*. As expected, Ehrlich ascites tumor cells harvested from insulin-treated hypoglycemic mice showed an increase in glucose carrier content comparing with control group while Ehrlich ascites tumor cells harvested from streptozotocin-treated hyperglycemic mice showed a reduction

in glucose carrier content comparing with untreated mice. The differences are statistically significant. In addition, injection of insulin to streptozotocin-induced diabetic mice resulting in reversal of hyperglycemia showed a restoration of glucose carrier on Ehrlich ascites tumor cells harvested from this group (Table 4). Again, a good correlation between serum glucose with the number of glucose carriers on different groups of tumor cells can be seen (Fig. 25). However, the results of this experiment cannot conclusively prove the direct effect of serum glucose on glucose carrier density in Ehrlich ascites tumor cells. Many workers (Suzuki and Kono 1980; Carter-Su *et al* 1981; Karnieli *et al* 1981; Kono *et al* 1981; Resh 1982) showed that insulin can increase the glucose uptake as well as glucose carrier in adipocytes due to translocation of glucose carrier from microsomal fraction to plasma membrane. Therefore the results of the present study on Ehrlich ascites tumor cells can be alternatively interpreted that the changes in glucose carrier on Ehrlich ascites tumor cells harvested from different groups of mice are caused by the excess or deficiency of insulin.

In order to differentiate these two possibilities, the direct effect of insulin on glucose carrier density in Ehrlich ascites tumor cells was studied. During tumor development, hypoglycemia caused by the development of tumor cells might stimulate the secretion of glucagon and glucocorticoid. In addition, it has been reported that steroid hormones like glucocorticoid, corticosterone and its metabolite, cortisone can decrease the glucose transport by human erythrocytes (Lacko, Wittke and Geck 1975) at a competitive manner. However the

mode of action of steroids on biological membrane at the molecular level, for examples their effects on the glucose carrier density and glucose uptake rate of tumor cells are still obscure. Therefore it is of interest to find out whether glucagon and glucocorticoid increase or decrease the glucose carrier of the tumor cells. Since the injection of these hormones to tumor bearing mice may complicate the serum glucose concentration, the effect of steroid hormones and insulin on the 7 day-old Ehrlich ascites tumor cells were studied *in vitro*. The results of the present study showed that insulin even at a high concentration of 20 µg/ml cannot stimulate the increase in glucose carrier on Ehrlich ascites tumor cells. However, both the glucagon and glucocorticoid can decrease the density of glucose carrier in the tumor cells statistically significantly by about 35%. The finding that Ehrlich ascites tumor cells show response to glucagon and glucocorticoid but not to insulin is difficult to explain. The transformation from normal cells to malignant cells usually leads to a de-differentiation and loss of a variety of cell apparatus including some hormone receptors resulting insensitivity to such hormones (Warburg 1956) and the result of present study may suggest the loss of insulin receptor in Ehrlich ascites tumor cells. Nirenberg (1959) show that Ehrlich ascites tumor cells lack to a large extent of hormonal regulatory mechanism may be through the loss or inhibition of particular enzyme pathways. However, Ginsberg *et al* (1982) reported that insulin receptors are present on Ehrlich ascites tumor cells and these receptors

are biologically active. Pavelis *et al* (1979) showed that the levels of insulin-like (glucose-lowing) substances in the cell free extract from the Ehrhich ascites tumor cells are higher than these in muscle extract indicating that insulin may be required for tumor growth. On the other hand, Crane *et al* (1956) also showed that insulin cannot show any stimulatory effect on glucose uptake by Ehrlich ascites tumor cells and our results are consistent with their finding. These may be a compromised explanation that insulin effect on Ehrlich ascites tumor cells may not be responsible for glucose uptake but for other activity such as amino acids uptake or their incorporation into proteins. The exact effect of insulin on Ehrlich ascites tumor cells awaits further study. The decrease in glucose carrier content in Ehrlich ascites tumor cells caused by glucagon and corticosterone is surprising. Since the uptake of glucose is essential for the proliferation of tumor cells, the decrease in glucose carrier can lead to a decrease in glucose uptake and thus to a decrease in tumor proliferation. However, the characteristics of binding sites (receptors) for these hormones on Ehrlich ascites tumor cells is still not clear. Further studies in order to answer these questinos are needed.

In order to further confirm the effect of extracellular glucose concentration on glucose carrier density in Ehrlich ascites tumor cells, effect of different glucose concentrations on 7 day-old Ehrlich ascites tumor cells were studied *in vitro*. As the glucose in medium was metabolized by the tumor cells, glucose concentration decreased progressively during the

incubation. High glucose concentration in medium can decrease the glucose carrier content in Ehrlich ascites tumor cells. The repression of glucose carriers by high content of glucose are reported in other cell lines (Maryineau *et al* 1972; Kalckar and Ullrey 1973; Hatanaka 1973; Shaw and Amos 1973; Kletsien and Perdue 1975; Salter and Cook 1975; Venuta and Rubin 1975; Salter ans Cook 1976; Christopher, Colby and Ullrey 1976; Musliner, Chrousos and Amos 1977). In high glucose group, when the glucose concentration reached a critical lower level, the Ehrlich ascites tumor cells were found to restore their glucose carrier density (Fig. 27). On the other hand, in the low glucose group, when the glucose in the medium was used up at 8 hours after incubation, the tumor cells began to increase their glucose carrier at 12 hours after glucose starvation. The derepression of glucose carriers are also reported in other cell lines (Amos *et al* 1977; Musliner, Chrousos and Amos 1977; Christopher, Colby and Ullrey 1976; Salter and Cook 1976)

The time lag for Ehrlich ascites tumor cells to increase the glucose carrier after the prolonged glucose starvation may suggest the involvement of protein synthesis in this respect. It should be noted that at 2 hours after incubation, a small drop in glucose carrier was observed in both high glucose and low glucose groups, and this drop in glucose carrier may represent the adaption of tumor cells to the medium from peritoneal cavities of mice because the reduction in glucose carrier is nearly identical in both two groups.

The above results strongly suggest that repression and

derepression of glucose carrier in response to glucose may appear during Ehrlich ascites tumor cells development *in vivo*.

Therefore we postulate that when inoculated Ehrlich ascites tumor cells were first exposed to the peritoneal fluid of normal mice, where the glucose content was about 95 mg/100 ml, so their glucose carrier number decrease by the regulation of repression. However during tumor development, the glucose level in ascites fluid is undetectably low. Tumor cells may have to increase their glucose carrier for facitilated uptake of glucose. As serum glucose decrease progressively, the tumor cells have to increase more their glucose carrier number. This may be considered as a compensatory mechanism for glucose uptake. This hypothesis is proven by the repression and derepression of glucose carrier according to the changes in glucose concentration *in vitro*. However other factors being responsible for the changes in glucose carriers on tumor cells may not be ruled out. Lawrence and Jullien (1980) reported that Rous sarcoma virus-transformed cells can release a factor that can increase hexose transport by the transformed cells, and Todaro and Larco (1978) showed that sarcoma-virus transformed cells can produce a growth factor. Since increase in net body weight of Ehrlich ascites tumor bearing mice was observed at early state (Fig.2), and this increase in body weight may be due to the factors with growth stimulating effect and these factors may also influence the number of glucose carrier in the tumor cells. However whether Ehrlich ascites tumor cells can produce and release a factor (or factors) that can stimulate its proli-

feration and glucose uptake is not known. Furthermore, Bernard *et al* (1982) reported that during embryo development, the number of glucose carrier increased in chick fibroblasts. The increase in glucose carrier on Ehrlich ascites tumor cells may be just a result of tumor development which is independent of other factors.

VI. Effect of methorexate (MTX) on the glucose uptake and glucose carrier on Ehrlich ascites tumor cells *in vivo*

It has been reported that methotrexate arrasts the growth of Ehrlich ascites tumor cells by inhibiting nucleotide synthesis and induction of a "purineless" state in cells (Hryniuk 1973). The cytotoxic efficiency is dependent on the schedule of administration. Early administration was found to be more effective (Table 7), an observation consistent with the cell cycle-specific nature of methotrexate action (Ernst 1971) and the known relative preponderance of resting cells in aged tumor (Lala and Patt 1966). MTX also reduced the glucose uptake rate and the number of glucose carrier in Ehrlich ascites tumor cells (Table 8 and Table 9). The efficiency of the drug in this respect roughly paralleled its efficiency in reducing tumor size. In this connection, it should be noted that glucose carrier on Ehrlich ascites tumor cell is a protein (Cuppoletti, Mayhew and Jung 1981). MTX-induced depletion of nucleotide and the consequent inhibition of DNA, RNA and protein synthesis might have constituted primary chain of events leading to reduced glucose carrier production. The reduction of the glucose uptake rate

may be due directly to the reduction in glucose carrier.

VII. Scope for further study

The present study provides more understanding to the characteristics of glucose carrier on Ehrlich ascites tumor cells. Since this carrier molecule has just been partially purified (Cuppoletti, Mayhew and Jung 1981), its molecular structure is still not clear. Nature of the binding of cytochalasin B and other sugars to this carrier molecule also remains unknown. Deves and Krupka (1978) suggested that cytochalasin B asymmetrically bound to human erythrocytes and Ginsburg (1978) suggested that two simple asymmetric antiparallel carriers with different K_M were responsible for hexose transport. In mammalian cell lines including human Hela cells, mouse L- and P-388 leukemia cells, Chinese hamster ovary cells and Novikoff rat hepatoma cells, hexose transport system was found with broad substrate specificity and differential molibilty of loaded and empty carrier, but with directional symmetry (Graff, Wohlhueter and Plagemann 1981; Plagemann *et al* 1981). Studies on glucose carrier on Ehrlich ascites tumor cells in this approach is also of interest.

Translocation of glucose carrier from microsomal pool to plasma membrane was seen in adipocytes upon insulin stimulation. Insulin was found without effect in the stimulation of glucose carrier, however, whether translocation of glucose carrier occurs in Ehrlich ascites tumor cells is not known and the factor(s) and mechanism which stimulate translocation of glucose carrier, if occurs, is also not understood.

Although glucose starvation is strongly suggested to be responsible for the changes in glucose carreir on Ehrlich ascites tumor cells, the mechanism at molecular level of glucose carrier repression and derepression caused by glucose level is still obscure. To answer the above questions, further studies on the nature of carrier on Ehrlich ascites tumor cells are required.

Recently, several workers showed that anti-human erythrocyte glucose transporter antibody (Baldwin and Lienhard 1980) can cross-react with the glucose transporer of rat adipocyte, Rous sarcoma virus-transformed chick embryo fibroblasts, human Hela cells and mouse L-1210 cell (Wheeler *et al* 1982; Salter *et al* 1982; Sogin and Hinkle 1980) and thus provide a new dimension for the study in glucose transporter. Lienhard *et al* (1982) made use of this technique to identify the glucose transport with reponsiveness to insulin of rat adipocyte. Since there are similarities in the nature of glucose transporter shared by Ehrlich ascites tumor cells and other cell lines, the antibody against human erythrocyte glucose transporter may also cross-react with the glucose carrier on Ehrlich ascites tumor cells and thus provide a new technique for the study on the characteristics of glucose carrier of the tumor cells.

Most recently, glucose carriers on human erythrocytes can be photoaffinity labeled with ^{3}H-cytochalasin B (Carter-Su *et al* 1982; Shanaha 1982). This new tecnique may be applicable in the further studies on the nature and characteristics of glucose carriers on Ehrlich ascites tumor cells.

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